RELATIONSHIP BETWEEN PROTEIN-BINDING AND FREE DRUG CONCENTRATIONS OF A SPARINGLY WATER-SOLUBLE SELECTIVE SEROTONIN REUPTAKE INHIBITOR (ESCITALOPRAM) AND ITS INTERACTION WITH ARSENIC

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Abstract. Escitalopram is the (S)-enantiomer of the racemic selective serotonin reuptake inhibitor antidepressant citalopram. Clinical studies have shown that escitalopram is effective and well tolerated in the treatment of depression and anxiety disorders. Single isomers of the selective serotonin reuptake inhibitors citalopram (escitalopram, S-citalopram) and fluoxetine (R-fluoxetine) are currently under development for the treatment of depression and other psychiatric disorders. Previous studies conducted in laboratory animals have revealed that the biological effects on serotonin reuptake for citalopram reside in the S enantiomer. In contrast, both enantiomers of fluoxetine contribute to its biological activity. The protein-binding of escitalopram was measured by equilibrium dialysis in the bovine serum albumin (BSA). Free escitalopram concentration was increased due to addition of arsenic which reduced the binding of the compounds to BSA. During concurrent administration, arsenic displaced escitalopram from its high-affinity binding Site I, and free concentration of escitalopram increased from 8.32% to 24.51% and 0.067% to 14.56% of Site I probe respectively. Thus, it can be ascribed that arsenic displaced escitalopram from its binding site resulting in an increase of the free escitalopram concentration in plasma. Potential correlations of these unique attributes of escitalopram and Arsenic are discussed.

Key words: Arsenic; escitalopram, bovine serum albumin, plasma protein binding, drug interaction, antidepressants, SSRIs.

INTRODUCTION

The selective serotonin reuptake inhibitor (SSRI) citalopram is a racemate that comprises an S(+)-enantiomer (escitalopram) and an R(-)-enantiomer (R-citalopram) in a 1:1 ratio. In studies using the individual enantiomers, the S-enantiomer has been shown to be responsible for essentially all the serotonin

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reuptake inhibition [4] and non-clinical antidepressant activity [3] of citalopram. In terms of serotonin reuptake inhibitor activity, R-citalopram was about 150 times less potent than escitalopram in an in vitro rat brain synaptosome system [4, 11]. Escitalopram oxalate (S-citalopram), a selective serotonin re-uptake inhibitor antidepressant which is the S-enantiomer of citalopram, is in clinical development worldwide for the treatment of depression and anxiety disorders. Preclinical studies demonstrate that the therapeutic activity of citalopram resides in the S-isomer and that escitalopram binds with high affinity to the human serotonin transporter. Efficacy has also been shown in treating generalised anxiety disorder, panic disorder and social anxiety disorder. The pharmacokinetic properties of exogenous and endogenous compounds can be influenced by reversible binding to human serum albumin (HSA), which is thought to be one of the primary determinants of the pharmacokinetic properties of drugs [2, 5, 6, 9].

Arsenic presents in the environment both in organic and inorganic form. It is mainly used as insecticides, fungicides and herbicides. Arsenic works through coagulation of proteins, complexation with co-enzymes, and uncoupling of phosphorylation. By inhibiting enzymes containing vicinal groups at their active center, arsenic shows its acute toxicity [8]. Arsenate disrupts oxidative phosphorylation when phosphate is substituted and then ATP is formatted [1]. This results in depletion of cellular energy stores. Human body can detoxify inorganic arsenic through methylation that is then excreted in the urine [17]. Arsenic has a high affinity to deposit in the tissues, nail, hair and some protein for a long time. Before methylation inorganic arsenic perhaps binds with tissue protein [19] and it occurs mainly in the liver [7].

MATERIALS AND METHODS

DRUGS AND REAGENTS USED IN THE EXPERIMENT

Escitalopram (Insepta Pharmaceutical Ltd., Bangladesh) warfarin, diazepam, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), cellulose nitrate membrane (Medicell International Ltd., Liverpool Road, London, UK; molecular wt 1,200 Daltons), bovine serum albumin (BSA) (fatty acid-free, fraction V, molecular wt 66,500 from Sigma Chemical Ltd., USA**)**, arsenic trioxide (As_2O_3), and sodium arsenate.

INSTRUMENTS USED

The following instruments were used: p^H Meter (HANNA Microprocessor p^H Meter, Portugal), SP8-400 UV/VIS Spectrophotometer (Thermospectronic, England), Metabolic Shaking Incubator (Clifton Shaking Bath, Nical electro Ltd., England), and Micro Syringe (well. Liang. Jin. Yang.q.I., China.)

METHOD USED

Equilibrium dialysis was employed in the study [12, 13].

SITE-SPECIFIC PROBES METHOD

We have used different site-specific probes to enhance our understanding of the drug-BSA interaction and thereby characterization of binding sites of the drugs used in the study on the BSA molecule [2, 12, 13, 15, 16].

Two site-specific probes were used. These are: (a) warfarin sodium (Site I-specific probe) and (b) diazepam (Site II-specific probe) that may be used for the identification of the binding sites of the drugs on BSA. In the direct procedure, the ratio of BSA and probe (either warfarin or diazepam) was 1:1 (2×10^{-5} M: 2×10^{-5} M), and different concentrations of drug were added. In the reverse procedure, the ratio of BSA and drug was 1:1 (2×10^{-5} M: 2×10^{-5} M), and different concentrations of probe (Site I-specific warfarin sodium or Site II-specific diazepam) were added. After conducting equilibrium dialysis, the free concentration of probe will be determined in direct procedure.

PREPARATION OF STANDARD CURVE

Standard curve has been prepared using various concentrations and their corresponding concentration at pH 6.4, 7.4 and 8.4. UV spectrophotometric scanning of the drugs-escitalopram, diazepam, and warfarin-showed maximum absorbance of the UV light at 238 nm, 235 nm, and 308 nm respectively. Escitalopram showed linearity at a concentration range of 10–80 μM/mL with a confidence level of 0.9976, 0.9995 and 0.9973 at pH 6.4, 7.4 and 8.4 with linear equation ($Y = 0.1899\ X + 0.0126$), ($Y = 0.1483\ X + 0.0028$) and ($Y = 0.1881\ X + 0.0239$) respectively. A similar standard curve was also prepared both for diazepam and warfarin, and the concentrations of those drugs were calculated using the corresponding linear equations.

ESTIMATION OF ASSOCIATION CONSTANT OF ESCITALOPRAM AT pH 6.4, 7.4 AND 8.4 AT 27 ºC TEMPERATURE

Estimation of the association constant of escitalopram was done at pH 6.4. Ten clean and dried test tubes were taken, and 3 mL of previously-prepared 2×10^{-5} M BSA solution at pH 6.4 was taken in each of them. Escitalopram stock solution (1×10^{-2} M or 1×10^{-3} M) was added in different volumes to nine of the 10 test-tubes to have the following concentrations: 0.5×10^{-5} M, 1×10^{-5} M, 2×10^{-5} M, 3×10^{-5} M, 4×10^{-5} M, 5×10^{-5} M, 6×10^{-5} M, 7×10^{-5} M, 8×10^{-5} M, and 9×10^{-5} M. The tenth test-tube containing only BSA solution at pH 6.4 was marked as ‘control’. After mixing

the solutions, these were allowed to stand 10 minutes for maximum binding of escitalopram to BSA; 2 mL from each test-tube was pipetted out and poured onto previously-prepared semi-permeable membrane-tubes and, finally, both sides of the tubes were clipped properly so that there was no leakage. The membrane-tubes containing the drug-protein mixture were immersed in ten 50 mL flasks containing 30 mL of phosphate buffer solution of pH 6.4. The mouths of the flasks were covered by foil-paper. These conical flasks were then placed in a metabolic shaker for dialysis for 10 hours at 27 ºC and 20 rpm. Buffer samples were collected from each flask after complete dialysis. Free concentrations of escitalopram were measured by a UV spectrophotometer at a wavelength of 238 nm. This similar procedure was used to determine the association constant of escitalopram at pH 7.4 and 8.4 the only exception the pH of the buffer solution.

DETERMINATION OF BINDING SITE OF ESCITALOPRAM USING WARFARIN SODIUM AS A SITE I-SPECIFIC PROBE

To determine the binding site of escitalopram, using warfarin sodium as a Site I-specific probe, the following successive steps were followed. From the previously-prepared 2×10^{-5} M BSA solution, 3 mL was taken in each of the eight cleaned and dried test-tubes; 1×10^{-3} M warfarin solution was added to seven of the eight test-tubes, and the final ratio of protein and warfarin was 1:1 (2×10^{-5} M: 2×10^{-5} M) in each of these seven test-tubes. The eighth test-tube containing only BSA solution was marked as ‘blank’ or ‘control’. These mixtures were allowed to stand for 10 minutes for allowing binding of the warfarin to its particular binding site. Escitalopram solution (either 2×10^{-2} M or 2×10^{-3} M) was added with increasing concentrations into six of the seven test-tubes containing 1:1 mixture of protein-warfarin. The final ratios of protein: warfarin: escitalopram were 1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5, and 1:1:6. The remaining test-tube contained only protein-warfarin mixture (1:1). After pipetting, the solution was properly mixed and allowed to stand for 10 minutes to ensure maximum binding of escitalopram to Site I and thereby displacing the probe from Site I on BSA. From each test-tube, 2 mL solution was taken into eight different semi-permeable membrane-tubes. The two ends of the membrane-tubes were clipped to ensure that there was no leakage, and the rest of the experiment was done as described above using phosphate buffer solution of pH 7.4.

DETERMINATION OF BINDING SITE OF ESCITALOPRAM USING DIAZEPAM AS A SITE II-SPECIFIC PROBE

To perform this experiment, the similar protocol has been followed successively using diazepam solution instead of warfarin. At the end of dialysis, samples were collected from each flask. The free concentrations of diazepam were

measured using a UV spectrophotometer at a wavelength of 238 nm (INN). Reverse experiment was also being conducted followed by a similar protocol by adding diazepam in an increasing concentration and measuring the free escitalopram concentration by spectrophotometer.

EFFECT OF ARSENIC ON ESCITALOPRAM-BINDING TO BSA IN PRESENCE OF SITE I-SPECIFIC PROBE WARFARIN-SODIUM

Previously-prepared 2×10^{-5} M BSA solution and 1×10^{-2} M warfarin solution, 2 mL and 12 mL, respectively, were added to each of the seven cleaned and dried test-tubes. The final ratio between protein and warfarin was 1:1 (2×10^{-5} M: 2×10^{-5} M) in each of seven test-tubes so that Site I is sufficiently blocked by warfarin–sodium. The seventh test-tube containing only BSA solution was marked as "blank". After that, escitalopram was added in six of the seven test-tubes, and protein-warfarin- escitalopram ratio was 1:1:1 (**2×10^{-5}** M : 2×10^{-5} M : 2×10^{-5} M). Arsenic was added with an increasing concentration into five of the six test-tubes containing 1:2:1 mixture of protein-warfarin-escitalopram to make the final ratio of protein-warfarin- escitalopram arsenic 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:4, 1:2:1:6, 1:2:1:8, 1:2:1:10. Arsenic was not added to one test-tube. The solutions were then properly mixed and allowed to stand for 15 minutes for the confirmation of maximum binding to BSA. After that, the solution was pipetted out and poured into seven semi-permeable membrane-tubes. The two ends of the membrane-tubes were clipped to ensure that there was no leakage, and the rest of the experiment was done as described above using phosphate buffer solution of pH 7.4.

EFFECT OF ARSENIC ON ESCITALOPRAM-BINDING TO BSA IN ABSENCE OF SITE I-SPECIFIC PROBE WARFARIN-SODIUM

To perform the experiment, the previously-described procedure was followed successively in absence and presence of warfarin sodium. Arsenic was added with an increasing concentration to five of the six test-tubes containing 1:1 mixture of protein- escitalopram to make the final ratio of protein: escitalopram: arsenic 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:6, 1:1:8, 1:1:10. Arsenic was not added to the first test-tube which contained only protein-escitalopram mixture (1:1). At the end of dialysis, samples were collected from each flask. The free concentrations of escitalopram were measured by a UV spectrophotometer at a wavelength of 238 nm (INN).

RESULTS

Both association constant (k_a) and number of binding sites (n) of escitalopram were determined using the Scatchard plot. To estimate the binding parameters of escitalopram, equilibrium dialysis (ED) was used and the subsequent non-linear

shape of the Scatchard plot describes both high- and low-affinity binding sites of drugs on protein molecule.

DETERMINATION OF ASSOCIATION CONSTANT AND NUMBER OF BINDING SITES

Escitalopram was characterized by a high-affinity association constant (k1) to BSA and the values at pH 6.4, 7.4 and 8.4 were (21.612 ± 1.347, 10.557 ± 0.398 and 30.278 ± 1.373) × 10^{-5} M respectively (represented in Table 1) while the low-affinity association constants (k_2) for escitalopram were found (12.327 ± 0.814, 1.910 ± 0.148 and 16.579 ± 0.521) × 10^{-5} M. For this drug, the number of high-affinity and low-affinity binding sites were (1.367 ± 0.115, 1.633 ± 0.058 and 1.213 ± 0.040) × 10^{-5} M and (3.277 ± 0.025, 4.817 ± 0.076 and 4.987 ± 0.186) × 10^{-5} M respectively at pH 6.4, 7.4 and 8.4. In the case of escitalopram bound to BSA, the high-affinity association constant (k_1) was found to increase when pH was changed from 6.4 to 8.4, but it only decreased at pH 7.4 (represented in figure 1). The similar circumstances were observed in low-affinity association constant (k_2) (Fig. 2). When the changes of physiological pH occur, BSA undergoes conformational alteration, which is generally termed N-B transition. BSA remains almost entirely in neutral form at pH 6 and in basic form at pH 9. When the protein is in the B-conformation, fewer protons are bound to BSA than in the N-conformation. Thus, the high-affinity and low-affinity binding of escitalopram is affected by change in pH. These differences in effect of pH may be due to the structural modification of protein molecule and, for this reason, at a given pH value, the binding site for escitalopram is more suitable or properly accommodated, while at other pH values, the binding sites become less convenient and less accommodating to the drugs in concern.

Table 1

Parameters of escitalopram bound to BSA at different pH values

pH	Association constant		No. of binding sites	
	k_1 (high affinity) × 10^{-5} M	k_2 (low affinity) × 10^{-5} M	n_1 (high affinity) × 10^{-5} M	n_2 (low affinity) × 10^{-5} M
6.4	21.612 ± 1.347	12.327± 0.814	1.367± 0.115	3.277± 0.025
7.4	10.557± 0.398	1.910± 0.148	1.633±0.058	4.817±0.076
8.4	30.278± 1.373	16.579± 0.521	1.213± 0.040	4.987±0.186

Values represent three consecutive experiments and are expressed as mean±standard error.

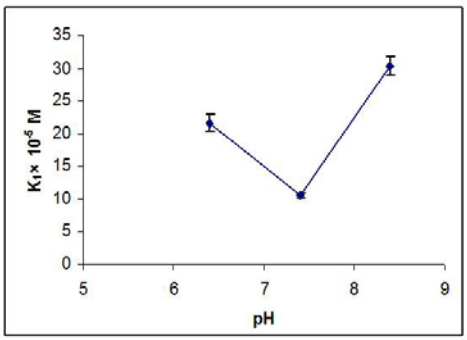

Fig. 1. Effect of pH on high affinity association constant of escitalopram oxalate bound to BSA.

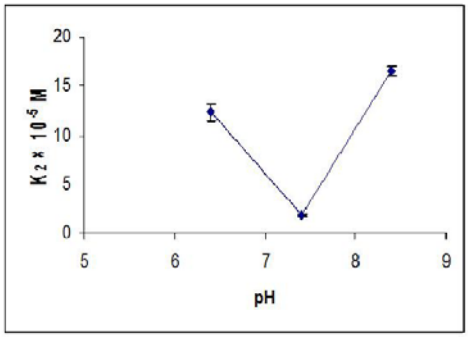

Fig. 2. Effect of pH on low affinity association constant of escitalopram oxalate bound to BSA.

DETERMINATION OF BINDING SITE

Well-established probes, which are specific for particular sites on the albumin molecule, are used for the identification of the binding site of the drugs on the protein molecule. If a drug is able to displace a probe from its binding site, it is assumed that the drug also binds to that particular site. Thus, the binding site and specificity and relative strength of binding to albumin of escitalopram have been determined by this principle. Here, as Site I-specific probe, warfarin-sodium and as Site II-specific probe, diazepam was used. To characterize the binding site of escitalopram, the free concentration of warfarin-sodium (Site I-specific probe) bound to BSA was measured upon the addition of escitalopram. It was found that the free concentration of warfarin-sodium increased from 0.791139% (as % of initial) to 37.97468% when the ratio of escitalopram to BSA was increased to 6 (Fig. 3). In contrast, under the same experiment conditions when, in lieu of warfarin-sodium, diazepam was used as Site II-specific probe, the increment of the free concentration of diazepam by escitalopram was from 7.912688% (as % of initial) to 35.19782% (Fig. 3). From these data, this is evident that, upon addition of escitalopram the increment of free concentration of warfarin-sodium is obviously little higher than that of diazepam. Thus, it can be concluded that escitalopram has slightly more affection to binding Site I. Again, as the displacement of diazepam is quite pronounced, it can be also suggested that escitalopram, in addition to Site I, also binds to Site II on the BSA molecule but to a lower extent.

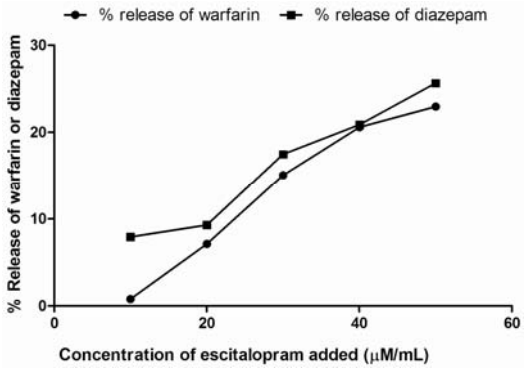

Fig. 3. Free concentration of warfarin and diazepam bound to BSA upon the addition of escitalopram at pH 7.4 and 27 ºC.

DISPLACEMENT OF ESCITALOPRAM DUE TO THE EFFECT OF ARSENIC

During concurrent administration of escitalopram and arsenic, site-to-site displacement takes place, and arsenic displaced escitalopram from its binding sites (Fig. 4). In the presence of probe, free concentration of escitalopram was more prominent. This displacement may be due to reduction of the binding site on bovine serum albumin. As observed from the model, during concurrent administration, arsenic displaced escitalopram from its high-affinity binding Site I. Thus, free concentration of escitalopram increased from 8.32% to 24.51% (Fig. 5) and 0.067% to 14.56% (Fig. 6) in absence and presence of Site I probe respectively.

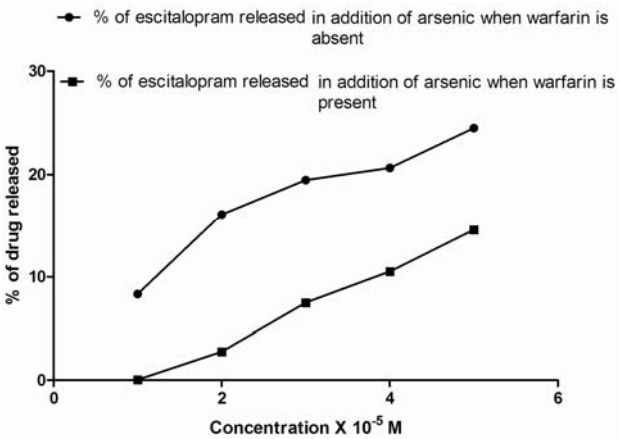

Fig. 4. Effect of arsenic on escitalopram binding to BSA in presence and absence of warfarin as site-I specific probe [BSA]: [warfarin] = 1:1 (2×10^{-5}M: 2×10^{-5}M).

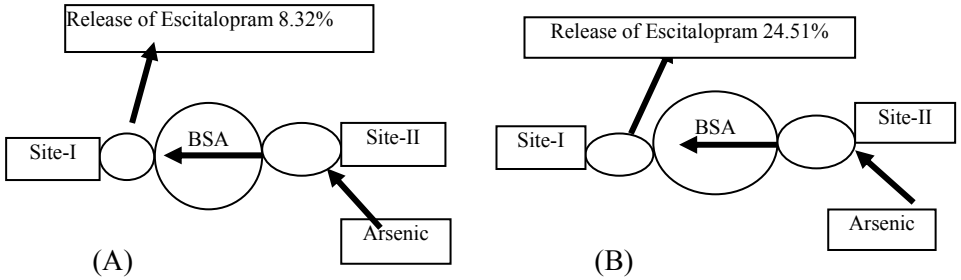

Fig. 5. Proposed models of the escitalopram-BSA-arsenic interaction in absence of warfarin (site-I specific probe). Concentrations were used: [BSA] = [escitalopram] = 2×10^{-5}M; [arsenic] =5×10^{-5}M; (A) normal initial condition of binding; (B) drug interaction in absence of probe.

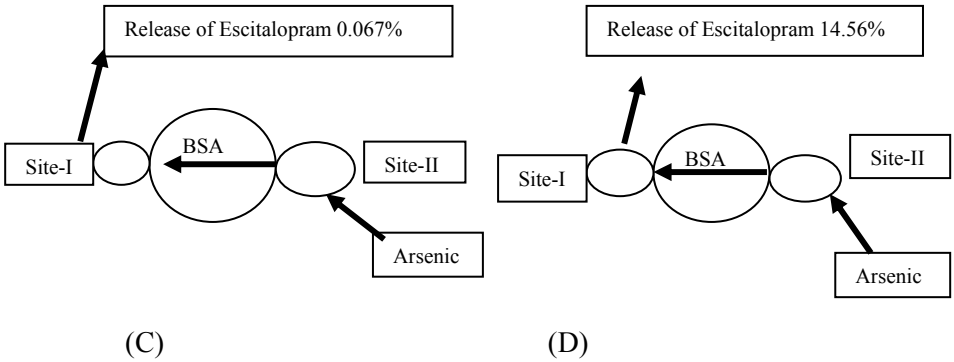

Fig. 6. Proposed models of the escitalopram-BSA-arsenic interaction in presence of warfarin (site-I specific probe). Concentrations used were: [BSA] = [escitalopram] = 2×10^{-5}M. [arsenic] = 5×10^{-5}M; (C) Normal initial condition of binding; (D) Drug interaction in presence of probe.

DISCUSSION

A recent study [10] has provided evidence of R-citalopram's allosteric effects at the human serotonin transporter, indicating that this enantiomer, far from being inert, introduces a conformational change in the transporter protein. These data support the theory that this conformational change interferes with the ability of the S-enantiomer (i.e. escitalopram) to inhibit serotonin uptake, and are thus consistent with the demonstrated clinical superiority of escitalopram over citalopram.

Metal compounds are distributed on earth crust and all over the environment. Humans can be frequently exposed to different metals such as chromium, nickel, arsenic, cadmium, and cobalt, at workplace and in usual household works. The people of some areas in Bangladesh are at high risk in arsenic contamination. To expose methylated trivalent arsenic can cause various side effects such as

inhibition of several key enzymes, damage to DNA structure, and activation of AP-1-dependent gene transcription [14]. Arsenic has a great affinity to binding site II and low affinity to binding site-I [18].

Displacement study was carried out during concurrent administration of two or more drugs. As there is strong analogy between BSA and HAS, it is assumed that similar types of binding characteristics will be exhibited by escitalopram when bound to HAS. Pharmacologic activity of a drug is sometimes related to its protein binding. When a drug shows less affinity for albumin due to any alteration of protein binding, then the pharmacologic effect of the drug may be significantly altered. But this is not always true, as the protein binding of a drug is not always indicative of its tissue distribution, its elimination or its activity. Therefore, precaution should be exerted, as pharmacological activity means not only the therapeutic activity but also it accounts for the toxic effects of a drug. However, from our limited data it is too early to draw such conclusion about the pharmacokinetic/ pharmacological properties of the drug. The concurrent administration of arsenic and the drug can alter the pharmacokinetics of escitalopram so care should be taken for prescribing escitalopram to the arsenic-affected people. Further research may reveal the exact mechanism of escitalopram interaction with bovine serum albumin in the presence of arsenic.

CONCLUSION

Earlier study results and new experimental data suggested that people who are exposed to arsenic frequently and suffer from anti depression along with arsenicosis, if intake drug like escitalopram may result in rapid action or rapid excretion or are associated with a variety of adverse effects that have a reflective impact on body mechanism even on cell proliferation. So take some extra precaution to prescribe escitalopram in arsenic affected areas' people.

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