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Short Communication

Column liquid chromatographic assay of the dopamine reuptake inhibitor vanoxerine (GBR 12909) in human serum

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ABSTRACT

A method for the assay of the dopamine reuptake inhibitor vanoxerine (GBR 12909, I) in human serum is described. Serum was diluted in urea (8 *M*) and extracted using C_{18} extraction columns. Compound I was eluted from the columns using methanol containing 1% (v/v) ammonia. The extracts were analysed by high-performance liquid chromatography with fluorescence detection. The limit of quantitation was 2 n*M*, corresponding to 1.04 ng/ml. Validation of the method showed acceptable recovery, accuracy, precision and specificity. The method has been used for drug assay in several clinical studies of the pharmacokinetics and therapeutic efficacy of compound I.

INTRODUCTION

Vanoxerine (GBR 12909) (I, see Fig. 1) is a selective dopamine reuptake inhib-

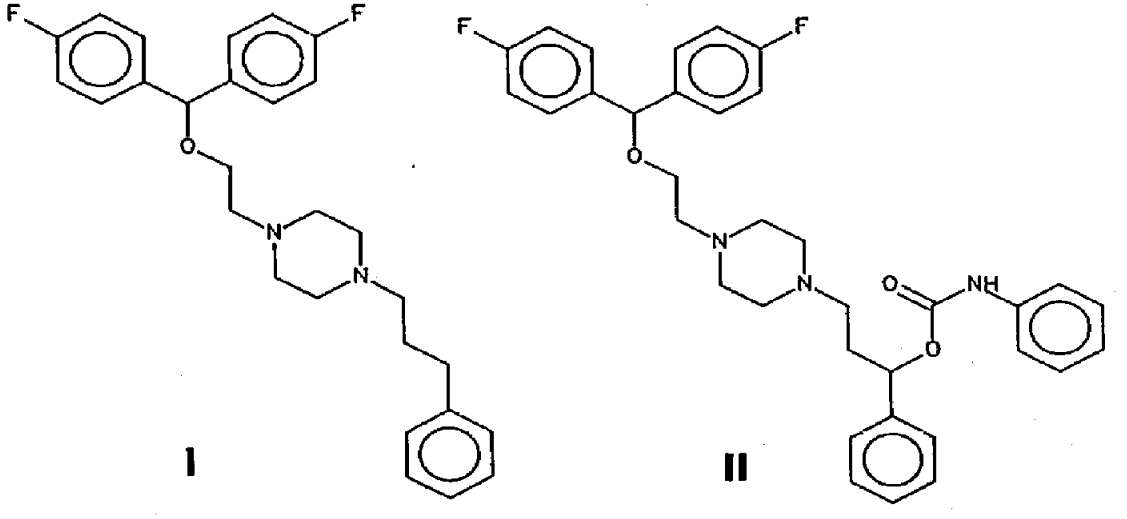

Fig. 1. Structure of the analyte vanoxerine (GBR 12909) (I) and of GBR 13461 (II) used as internal standard.

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itor with potentials for the treatment of depression and Parkinson's disease [1–3]. Compound I has previously been estimated in rat and dog serum by liquid–liquid extraction–gas chromatography (Gist Brocades, 1985, unpublished results) and in dog serum by liquid–liquid extraction–high performance liquid chromatography (HPLC) [4]. In the first clinical trials I was estimated in serum by liquid–liquid extraction–HPLC [5].

The method described here was developed in order to improve the sensitivity and precision of the assay. It was found that solid-phase extraction (SPE) using reversed-phase C_{18} columns is a precise and convenient procedure for sample preparation. However, special precautions had to be taken in order to achieve high recovery and make the method robust. The method has been used for drug assay in several clinical studies.

EXPERIMENTAL

Chemicals and reagents

I and GBR 13461 (II, used as internal standard, see Fig. 1) were synthesized by Novo Nordisk (Bagsvaerd, Denmark). Methanol of Chromoscan grade was obtained from Lab-Scan (Dublin, Ireland). Deionized water was purified by passage through a Milli-Q plant (Millipore). Analytical-grade perchloric acid (70–72%, v/v), hydrochloric acid and urea were obtained from Merck (Darmstadt, Germany). Bond-Elut C_{18} SPE columns (2.8 ml, containing 500 mg of stationary phase) were from Analytichem International (Harbor City, CA, USA).

Instrumentation

The HPLC system was purchased from Waters Assoc. (Milford, MA, USA). It consisted of a Model 6000A and a Model 510 solvent delivery system, a Model WISP 712 autosampler and a Model 470 spectrofluorometer equipped with a 5-μl standard flow cell. Excitation and emission wavelengths were set at 200 and 284 nm, respectively. Gain and filter were set at × 100 and 4 s, respectively. The emission bandwith was 30 nm. Data reduction was done using Maxima 820 software (Waters) installed on an IBM PC-AT microcomputer.

Elution of SPE columns was performed in a Minifuge T centrifuge (Heraeus, Osterode, Germany). Sample extracts were evaporated using a Speedvac SVC 200 H vacuum concentrator (Savant, Farmingdale, NY, USA) connected to a Model DUO 004B-016B vacuum pump (Pfeiffer, Asslag, Germany).

A Supelguard LC-8-DB (30 mm × 4.6 mm I.D., 5 μm particle size) served as the precolumn and a Supelcosil LC-8DB (75 mm × 4.6 mm I.D., 3 μm particle size) as the analytical column. Both columns were obtained from Supelco (Bellefonte, PA, USA).

Preparation of mobile phases, standards and spiked serum

Eluent A consisted of methanol–water (60:40, v/v). Eluent B was prepared by

adding 5 ml of perchloric acid (70–72%) and 7.5 ml of methanol to 1 l of eluent A. The final concentration of perchloric acid was 58 m*M*.

Stock solutions of I (277 μM) and II (100 μM) were prepared in eluent A containing in addition 1 m*M* hydrochloric acid. These stock solutions were used for preparing working calibrators and were stored at −18°C for up to one year. Calibrators were prepared in eluent A and contained 5–2000 n*M* analyte and in addition 2000 n*M* internal standard. They were stored at 4°C for up to two weeks. Internal standard for addition to serum samples contained 2000 n*M* II in eluent A.

Spiked serum for estimation of recovery, precision and accuracy was prepared by addition of 10–50 μl of 10 μM I in eluent A to a pool of serum from healthy volunteers to a total volume of 50 ml. Final concentrations of I were 2, 10 and 100 n*M*.

Assay procedure

Compound I was extracted from human serum by SPE. All elutions of extraction columns were performed by low-speed centrifugation (128 *g*, 18°C), except for the last washing with 60% (v/v) methanol, which was performed at 2000 *g* with slow acceleration. The columns were conditioned with 2 ml of methanol followed by 2 ml of water. A 0.5-ml aliquot of serum was pipetted directly onto the top of the column. A 0.1-ml aliquot of internal standard (2000 n*M* II in eluent A) was added followed by 0.5 ml of 8 *M* urea. The columns were subsequently washed with 2 ml of water and 2 ml of 60% (v/v) methanol in water, and analyte and internal standard were finally eluted from the column by 2 ml of methanol containing 1% (v/v) ammonia. Eluates were collected in conical glass tubes, and were evaporated to dryness in a vacuum concentrator. The dry extracts were redissolved in 100 μl of eluent A and 75 μl were injected into the HPLC system.

Chromatography was performed using a step gradient of perchloric acid. The initial mobile phase was 100% A, changing to 100% B at 4 min and back to 100% A at 14 min. The flow-rate was 1 ml/min and the run time was 18 min. Quantitation was done using peak heights.

RESULTS

Chromatographic separation

Fig. 2 shows chromatograms of a calibration standard prepared in eluent A, a blank serum extract, an extract of serum spiked with I and internal standard and an extract of a serum sample obtained from a human volunteer 80 min after an oral dosage of 125 mg of I.

As seen in Fig. 2A, analyte and internal standard with retention times of 6.1 and 8.6 min, respectively, were well separated using this procedure. During development of the method is was shown that increasing the methanol concentration of the eluents from 60 to 70% yielded higher signal-to-noise ratios while baseline

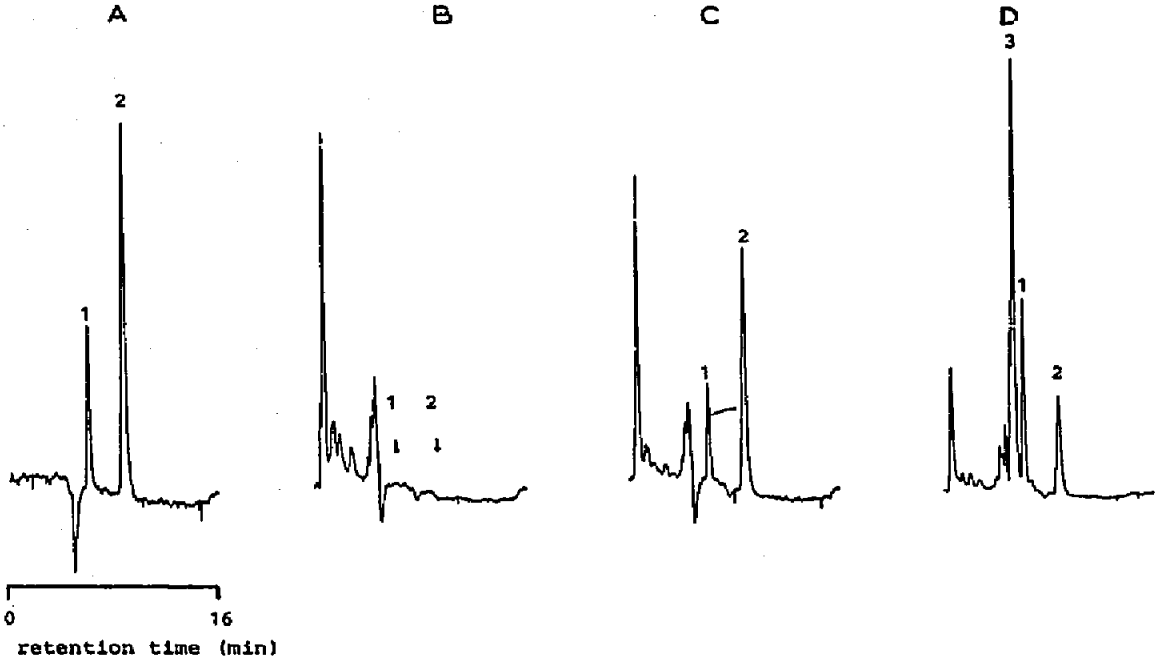

Fig. 2. (A) Chromatogram of a calibrator prepared in eluent A and containing 250 n*M* I and 2000 n*M* internal standard (II). A 75-*µ*l aliquot was injected onto the column. (B) Chromatogram of a blank serum extract. (C) Chromatogram of serum spiked with 50 n*M* I. (D) Chromatogram of serum obtained from a healthy volunteer 80 min after receiving an oral dose of 125 mg of I (concentration of I found to 218 n*M*). Peaks: 1 = I; 2 = II; 3 = metabolite(s) of I.

separation of drug and internal standard were maintained (results not shown). However, serum from human volunteers dosed orally with I showed the presence of several metabolites that were not fully separated from the parent drug. Thus, in order to improve the resolution, the methanol concentration of the eluents was reduced to 60%. As seen in Fig. 2D, metabolites and the parent compound were adequately separated using the present procedure.

The negative peak seen in front of the analyte was a system peak originating from impurities present in the eluents. With some lots of methanol, negative system peaks eluted close enough to the analyte to interfere with peak integration. This problem was solved by changing the time of inset of the perchloric acid step gradient, thereby changing the retention times of analyte and internal standard without affecting the system peaks.

Limit of quantitation, recovery, accuracy, linearity and precision

Serum spiked with 2, 10 and 100 n*M* GBR 12909 was assayed with six replicates at each concentration. The results from this experiment (Table I) were used to estimate limit of quantitation (LOQ), recovery, accuracy and linearity. LOQ was estimated as three standard deviations of the final assay results of serum spiked with 2 n*M* I (Table I). LOQ was estimated at 1.2 n*M*. For practical reasons

TABLE I

RESULTS OF THE INVESTIGATION OF SENSITIVITY, RECOVERY, ACCURACY, LINEARITY AND PRECISION FOR ASSAY OF I IN HUMAN SERUM

Spiked concentration of I (n*M*)	Recovery[a] (mean ± S.D.) (%)		Final assay results[b]			
	I	Internal standard	Individual (n*M*)	Mean (n*M*)	S.D. (n*M*)	C.V. (%)
2	104 ± 19	82 ± 3	2.9 1.9 2.8 2.2 2.8 2.3	2.5	0.41	16.3
10	82 ± 17	77 ± 8	8.6 10.6 11.7 12.0 9.1 11.0	10.5	1.38	13.1
100	69 ± 14	63 ± 13	108.4 107.2 109.3 107.5 113.5 108.3	109.0	2.31	2.1

[a] Results calculated individually for I and internal standard.
[b] Results for I calculated relative to the internal standard.

the lower limit of quantitation was set at 2 n*M*. The recovery of I ranged from 104% at 2 n*M* to 69% at 100 n*M*, and the recovery of internal standard ranged from 82 to 63% (all determinations at 2000 n*M* internal standard). The accuracy and linearity were estimated by least-squares linear regression of final assay results of spiked serum in the concentration range 2–100 n*M* I (Table I). The regression equation was $y = 1.09\,x - 0.04$. The standard deviation of residuals from the regression line ($S_{y\,.\,x}$) was 1.55 and the correlation coefficient was 0.999.

Intra-assay precision was estimated from the replicate assay results given in Table I. The coefficient of variation ranged from 16.3% at 2 n*M* to 2.1% at 100 n*M*. The between-assay variation was determined by assay once daily for ten days of human serum spiked with 10, 50 and 100 n*M* I. The coefficient of variation (C.V.) was 7.0, 4.9 and 3.4% at 10, 50 and 100 n*M*, respectively.

Selectivity

The selectivity of the assay was estimated in several ways. Firstly, blank serum extracts were investigated for endogenous peaks (Fig. 2B). Secondly, a number of drugs unrelated in chemical structure to I were investigated for interference with the chromatographic separation. These drugs included amitriptyline, amoxapine, clomipramine, fluphenazine, imipramine, isocarboxazid, mianserin, nortriptyline, opipramol, perphenazine, trifluoperazine, ranitidine, oxazepam, flunitrazepam, dihydroergotamine, paracetamol and orphenadrine. These drugs were injected onto the chromatographic column in solutions of 2 μM in eluent A. With the exception of orphenadrine, which coeluted with I, none of these drugs gave rise to chromatographic peaks (results not shown). The lack of detector response for these drugs was most probably due to the specificity obtained by fluorometric detection. However, as orphenadrine in a separate experiment was shown to be recovered in the serum extracts, serum samples containing this drug should not be asayed for I using the present method.

Thirdly, an attempt was made to verify the peak purity by running samples containing parent drug and metabolites using a UV photodiode-array detector instead of fluorescence. However, because of the poor UV absorption of these compounds this attempt did not succeed.

Another way of investigating the peak purity would be to assay pure metabolites in the chromatographic system. However, as the metabolic pattern of I has not yet been revealed, this approach was not applicable here. Instead, several potential metabolites were assayed. These included the *p*-hydroxy derivative, the benzylic hydroxy derivative and two N-dealkylated derivatives. None of these compounds interfered in the assay (results not shown).

Stability of calibrators and samples

Calibrators prepared in eluent A were stable for at least two weeks when stored at 4°C. The stability of I in serum was estimated using a serum pool spiked with 10, 50 and 100 nM I and stored in 2-ml samples at −18°C for eight months. No signs of deterioration during this time span were observed (results not shown).

DISCUSSION

Basic, lipophilic compounds often prove difficult to assay by reversed-phase liquid chromatography because of poor peak shape. I is one such example. An acceptable peak shape in this case was obtained by using a "deactivated" column packing developed for chromatography of basic compounds as well as by eluting the analyte and the internal standard from the column by means of a step gradient of perchloric acid. The perchloric acid in this case served a dual purpose: as an ionic pairing reagent and as an enhancer of the fluorescence, which is highly pH-dependent for this analyte, the fluorescence decreasing at increasing pH.

One advantage of the present method was the use of SPE on reversed-phase C_{18} extraction columns instead of extraction with organic solvents as used previously [4,5]. The recovery of more than 69% of the analyte achieved with SPE (Table I) was accomplished by diluting samples with urea prior to retention on the extraction columns. In the early experiments of assay development, when samples were diluted in water prior to retention and the analyte was eluted with pure methanol, the recoveries were rather poor: in general below 50%. As I is highly protein-bound, the poor recovery was thought to be the result of insufficient retention on the extraction columns. Thus, water was replaced with urea (8 *M*), which is known to denature proteins with loss of binding sites for smaller molecules [6], and this improved the recovery considerably. Later on it became obvious that even with this procedure recoveries varied considerably with different lots of extraction columns. This problem was solved by changing the solvent used to elute the analyte from the extraction columns from pure methanol to 1% (v/v) ammonia in methanol, which has been useful for other analytes, as well [7]. An examination of the mechanism of action of urea in the SPE procedure will be published in detail elsewhere.

The current method was shown to be sufficiently precise and accurate for assay of I down to 2 n*M* (corresponding to 1.04 ng/ml) in human serum. Although final proof of peak purity was not obtained because of poor UV absorption of the analyte, several potential metabolites did not interfere in the chromatography. As seen in Fig. 2D the bulk of metabolites, which have not yet been identified, eluted in front of the parent compound, although they were not individually separated. Furthermore, a battery of registered drugs were investigated for interference and, with the exception of orphenadrine, which coeluted with I in the chromatographic system, none of these drugs interfered.

In conclusion, the method described here was found to be well suited for assay of I in human serum. It has been used for drug assay in several clinical studies of the pharmacokinetics and therapeutic efficacy of I (to be published).

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