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SCREENING OF AMPHETAMINES BY GRADIENT MICROBORE LIQUID CHROMATOGRAPHY AND PRE-COLUMN TECHNOLOGY

K. SLAIS*, M. W. F. NIELEN**, U. A. Th BRINKMAN and R. W. FREI*
Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)

SUMMARY

Amphetamine-type drugs with a wide polarity range have been screened in both human and horse urine using on-line pre-concentration on pre-columns packed with hydrophobic and cation-exchange sorbents in series and gradient microbore high-performance liquid chromatography. The underivatized amphetamines were identified by UV detection at 210 nm. The method has potential for the automated liquid chromatographic screening of amphetamines in urine, *e.g.*, for doping control.

INTRODUCTION

Amphetamine-related drugs are often abused and misused in several kinds of sports. Forensic laboratories, hospital emergency-room laboratories and toxicology laboratories are frequently requested to analyse for their presence, especially in urine samples. Chromatographic methods available for amphetamine screening were recently reviewed[1,2].

Gas chromatographic analyses using a flame ionization detector[3,4] and off-line extraction have a detection limit close to 1 μg/ml of urine. The use of off-line solid-phase extraction, derivatization and selective nitrogen detection permits sensitivities down to several tens of pg/ml[5]. However, this method has been developed for the detection of metamphetamine only. A screening method based on liquid chromatography on bare silica[6] suffers from low selectivity of separation of the compound of interest from the sample matrix. To achieve a detection limit below 0.1 μg/ml of amphetamines in urine, high-performance liquid chromatography (HPLC) was combined with off-line extraction and derivatization[7]. Nevertheless, a selective, sensitive, rapid and automatable method for amphetamine screening is not yet available. Recently, automated liquid chromatographic methods using a combination of hydrophobic and ion-exchange pre-columns for the enrichment of anilines[8] and barbitu-

* On leave from the Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, 611 42 Brno, Czechoslovakia.
** Present address: MT-TNO, Department of Analytical Chemistry, P.O. Box 217, 2600 AE Delft, The Netherlands.

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rates[9] were developed. A similar approach was used in this work for the selective enrichment of amphetamines from the sample matrix.

The sorption properties of amphetamine-type drugs are based on the hydrophobic molecule moiety and the protonatable nitrogen atom. According to the literature[4], amphetamines can be adsorbed from water samples on reversed-phase sorbents at close to neutral pH. Consequently, the adsorption of amphetamines from untreated urine samples on a polymeric reversed-phase sorbent is suggested as the first step in the present analysis. The use of the polymeric reversed-phase sorbent instead of a silica-based sorbent maintains the high sorption capacity and prevents irreversible bonding of basic compounds on residual silanol groups. In order to use the basic character of amphetamines for their selective enrichment, the transfer of solutes from the hydrophobic pre-column to a cation-exchange pre-column was suggested as the next step.

The selective enrichment on pre-columns permits the use of low-selectivity but sensitive short-wavelength UV detection for the amphetamines. For example, on changing the wavelength from 257 to 210 nm, the molar absorptivity of the phenyl group increases 40-fold[10]. In this paper, we also suggest taking advantage of microbore HPLC[11]. Recently, microbore HPLC was successfully combined with pre-column sample enrichment[12,13]. To obtain the same solute concentration at the microbore column outlet, a smaller amount of sample is needed in comparison with the standard columns. In combination with on-line pre-column enrichment, it permits easier sample handling, filtration and pre-column flushing and therefore has the potential for a higher sampling frequency.

The method presented here is suggested for minimizing off-line sample treatment, allowing automation and screening for a wide range of amphetamine-type drugs in urine.

EXPERIMENTAL

Apparatus

The apparatus was constructed from two high-pressure pumps, four high-pressure switching valves, one solvent-selection valve, two pre-columns, a separation microcolumn, a UV detector and three capillary loops, as shown in Fig. 1. A laboratory-made syringe micropump with a volume of 50 ml was used to deliver the final solvent (strong eluting component of the mobile phase gradient) (E, Fig. 1).

An Altex (Berkeley, CA, U.S.A.) Model 110 pump together with a Rheodyne (Cotati, CA, U.S.A.) low-pressure six-port solvent-selection valve was used for pre-column flushing and mobile phase gradient preparation at 1 ml/min. Three laboratory-made high-pressure Rheodyne valves were used for pre-column switching, gradient preparation (in mixing loops) and sample introduction. A Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 UV detector equipped with a 0.5 μl flow cell was adjusted to 210 nm for monitoring the chromatograms and the gradient profile. The detector output was recorded on a Kipp & Zonen (Delft, The Netherlands) BD 8 recorder and evaluated manually.

Stationary phases and columns

Sample pre-concentration was accomplished on a 2 × 4.6 mm I.D. stainless-

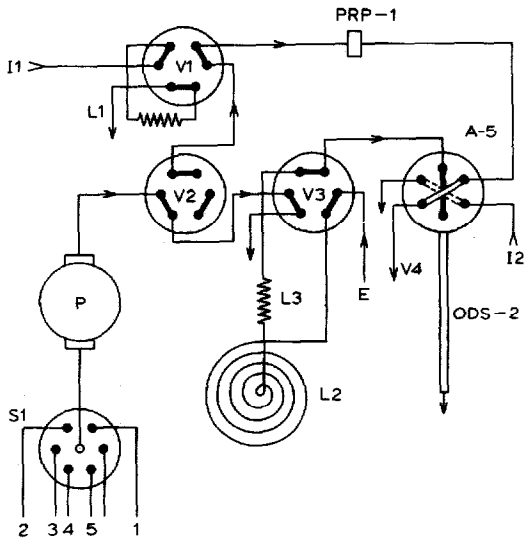


Fig. 1. Experimental set-up for the screening of amphetamines in urine according to the final procedure described in the text. P = pump; S1 = low-pressure selector valve; V1–V3 = high-pressure switching valves; V4 = switching valve with internal pre-column; PRP-1 = stainless-steel pre-column (2 × 4.6 mm I.D.) packed with PRP-1, 10 μm; A-5 = internal pre-column (4.5 × 1 mm I.D.) in V4 packed with Aminex A-5, 13 μm; ODS-2 = microbore column (100 × 1 mm I.D. packed with Spherisorb ODS-2, 5 μm; L1 = 1 ml stainless-steel capillary loop for urine sample; L2 = spiral coiled mixing capillary of stainless steel (800 × 1 mm I.D.) with coiling radius increasing uniformly from 2 to 30 mm; L3 = stainless-steel capillary (2000 × 0.25 mm I.D.) for adjusting isocratic part of gradient profile; I1 = injection of urine sample; I2 = injection of standard solution; E = strong eluting component of mobile phase gradient, 0.1 *M* sodium perchlorate–0.001 *M* perchloric acid, in methanol–water (50:50, v/v). 1 = Water; 2 = 0.001 *M* DMOA in 0.002 *M* perchloric acid; 3 = weak eluting component of mobile phase gradient, 0.1 *M* sodium perchlorate–0.001 *M* perchloric acid–0.01 *M* SOS in methanol–water (30:70, v/v); 4 = methanol–water (50:50, v/v); 5 = methanol.

steel pre-column laboratory-packed[14] using a micro-spatula with PRP-1 spherical 10 μm styrene–divinylbenzene copolymer (Hamilton, Reno, NV, U.S.A.). An Aminex (Bio-Rad Labs., Richmond, CA, U.S.A.) 13 μm pre-column (4.5 × 1.0 mm I.D.) was used as an internal pre-column in the laboratory-made switching valve described previously[10]. A valve void channel, of volume 5.7 μl, was used for on-column sampling of standard solution. The analytical microcolumn was a 100 × 1 mm I.D. glass-lined stainless-steel column laboratory packed with Spherisorb ODS-2, 5 μm (Phase Separations, Queensferry, U.K.).

Chemicals

Analytical-reagent grade perchloric acid and sodium nitrate and HPLC-grade methanol were obtained from J. T. Baker (Deventer, The Netherlands). Analytical-reagent grade sodium perchlorate was obtained from Merck (Darmstadt, F.R.G.), sodium dodecylsulphate from Sigma (St. Louis, MO, U.S.A.), sodium octylsulphonate (SOS) from Aldrich (Steinheim, F.R.G.) and N,N-dimethyl-*n*-octylamine (DMOA) from ICN Pharmaceuticals (Irvine, CA, U.S.A.). Demineralized water was purified in a Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.) to obtain

LC-grade water for use in mobile phases and standard solutions. Eluents were degassed in an ultrasonic bath under vacuum.

RESULTS AND DISCUSSION

Enrichment and cleaning procedure

As indicated in the Introduction, the adsorption of amphetamines on PRP-1 sorbent from untreated urine (pH close to 6) was chosen as the first step of sample enrichment. In order to achieve a high concentration of solutes in the detector, the volume of the sampling loop was chosen to be 1 ml. The breakthrough volume of ephedrine on the 2 × 4.6 mm I.D. PRP-1 pre-column was found to be 8 ml when pure water was used for the pre-column flushing. Therefore, 5 ml of water delivered by pump P (Fig. 1) was used for transfer of the sample from the sampling loop to the PRP-1 pre-column and pre-column clean-up. Using our experience with the enrichment of anilines[8], a 0.001 *M* solution of perchloric acid in pure water was tried for the transfer of the amphetamines to the Aminex A-5 cation-exchange pre-column. However, when using 5 ml of this solution only ephedrine and amphetamine were transferred and the less polar amphetamines remained on the PRP-1 pre-column. For quantitative transfer of fencamfamin (the last eluted compound of interest, see Table I) at least 25% (v/v) of methanol in the transfer solution was needed. However, in this instance, a large amount of matrix is transferred together with the amphetamines, which leads to a substantial increase in the background signal and thus to an increase in the detection limit.

The use of a competing ion in the transfer solution was suggested for the selective displacement of amphetamines from the PRP-1 pre-column. This suggestion follows the approach of transitory mobile phase environments described recently by us[15]. From neutral and acidic media, the amphetamines are adsorbed on a reversed-phase sorbent in their protonated form together with hydrophobic compounds of the sample matrix. By introducing a hydrophobic, positively charged ion into the mobile phase, substantial elution of positively charged solutes can be achieved via displacement mechanisms without influencing the retention of the neutral compounds[16]. A satisfactory transfer of the analytes from the PRP-1 to the Aminex cation-exchange pre-column was found to occur when the PRP-1 pre-column was flushed with 5 ml of 0.001 *M* DMOA in water (adjusted to pH 3 with perchloric acid). Under these conditions, even fencamfamin is transferred, although only with about a 30% recovery (*cf.*, Table II). It should be noted that the use of DMOA does not cause an increase in the background signal in comparison with a solution of perchloric acid. Higher concentrations (*e.g.*, 0.004 *M*) of DMOA led to a substantial decrease in the recovery of early eluting amphetamines and to a strong influence on the blank chromatogram. This observation indicates the breakthrough of DMOA and displacement of the adsorbed amphetamines from the Aminex A-5 pre-column. A lower concentration of a stronger displacing ion (*i.e.*, with a longer carbon chain) would probably lead to a higher recovery of the last eluting amphetamine without influencing the recovery of the other compounds of interest.

Gradient separation

Amphetamine-related drugs have a wide polarity range, as can be seen in Table

I. The only possibility of achieving the selective elution of compounds with a wide retention range is to use a mobile phase gradient. The device for the preparation of a mobile phase gradient compatible with microbore columns and automated systems described recently[17] was used in this work for the gradient elution of amphetamines. Amphetamines and interfering compounds such as catecholamines and indole derivatives (see Table I) occur in the mobile phase in their protonated form when using a mobile phase pH that is compatible with high-performance silica-based column packings, *i.e.*, pH 2–8. A useful mode of separation of ionized organic compounds is ion-pair reversed-phase chromatography[16], in which ionized solutes are adsorbed on the stationary phase due to both hydrophobic and electrostatic interactions. The retention of ionized compounds can therefore be varied by changing the nature and/or the content of the organic solvent and by changing the nature and/or content of the counter ion in the mobile phase.

Initially, a gradient from 0.1 *M* sodium perchlorate–0.001 *M* perchloric acid in methanol–water (10:90, v/v) to 0.1 *M* sodium perchlorate–0.001 *M* perchloric acid in methanol–water (40:60, v/v) was used to separate the amphetamines on the reversed-phase ODS-2 microbore column. In this instance, the concentration of perchlorate used as a weak pairing ion was kept constant while varying only the content of the organic modifier. A good separation of all amphetamines from interfering compounds (Nos. 1–5, see Table I) was achieved when standard solutions in water were injected directly on to the microbore column. However, when sampling was effected via adsorption and elution from the Aminex A-5 pre-column, very broad peaks of early eluting compounds were obtained even at elevated (3 *M*) concentrations of perchlorate. This was probably due to the hydrophobic nature of the polymeric matrix of the Aminex A-5 cation exchanger. The adverse influence of the Aminex A-5 pre-column on the amphetamine peak shape did not arise when using a methanol–water gradient from 40:60 (v/v) to 65:35 (v/v) at a constant concentration of 0.1 *M* sodium perchlorate and adding 0.001 *M* perchloric acid and 0.001 *M* sodium dodecylsulphate as a second counter ion. Narrow peaks and a reasonable separation of the amphetamines from interfering compounds were now observed. However, a poor separation of some of the amphetamines themselves was found. As an acceptable compromise, a gradient starting with methanol–water (30:70, v/v) containing 0.01 *M* SOS and ending with methanol–water (50:50, v/v) without any surface-active component was chosen; concentrations of 0.1 *M* sodium perchlorate and 0.001 *M* perchloric acid were maintained constant during the gradient. Omitting the pairing ion from the final mobile phase enabled the difference in methanol content between the initial and final eluents to be reduced which improved the baseline stability.

The gradient steepness and volume can be varied simply by adjusting the volume of initial solvent introduced into loops L2 and L3 (Fig. 1; see also ref. 17). The gradient shape obtained by introducing 0.5 ml of initial solvent was found to yield an acceptable compromise between analysis speed and analyte separation. The shape of the gradient used for all presented separations is shown in Fig. 2a. The retention times obtained for solutions of standards are listed in Table I, which includes data on the relative standard deviations of the retention times of the amphetamines.

A typical chromatogram of a gradient separation of some amphetamines with large differences in their polarity is shown in Fig. 2b. This model separation was also chosen for testing the reproducibility of the gradient separation. The influence of

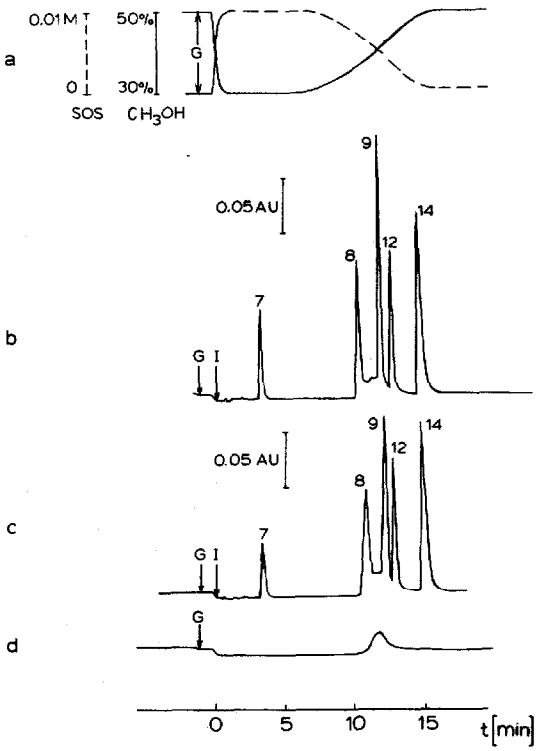


Fig. 2. Chromatographic profiles of gradient separation of standard solution. (a) Gradient shape obtained from open-tubular mixer[17] described in the text and in Fig. 1, 0.5 ml of weak eluting component of mobile phase gradient introduced into mixer within 0.5 min, gradient run at 1.2 μl/s, gradient traced by adding 0.0003 *M* sodium nitrate to the weak eluent and monitoring at 2 a.u.f.s., 210 nm; UV detector, Spectroflow 757. (b and c) Chromatograms of standard mixture separated in gradient described in Fig. 2a. Compound numbers (see Table I) and amounts of free base introduced (μg): 7, 0.41; 8, 0.94; 9, 0.84; 12, 0.86 and 14, 1.22. Sampling: (b) by void channel in V4; (c) by adsorption on Aminex A-5 pre-column in V4. G = start of the gradient. I (b) = on-column injection; I (c) = switching of Aminex A-5 pre-column to flushing on microcolumn. (d) Baseline course during gradient run; conditions as in (b) and (c); for other conditions, see (a) and Fig. 1.

sampling via adsorption on the Aminex A-5 pre-column on the separation and the peak shape follows from a comparison of Fig. 2b and c. Although in the latter instance the peaks are broader than in the former, the separation is still acceptable. The baseline of the UV detector during the gradient run can be seen in Fig. 2d.

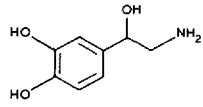
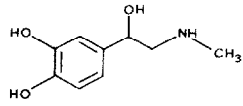
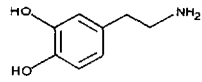
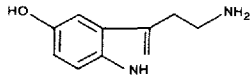
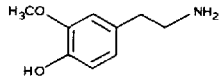
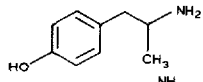
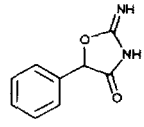
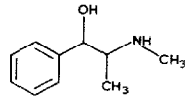
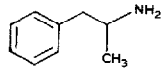
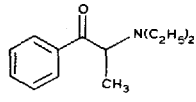
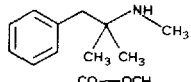
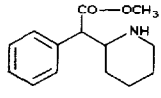
The possible interfering amines present in urine (Nos. 1–5, t_R = 0.6–2.6 min, see Table I) are eluted in the initial (isocratic) part of the chromatogram, whereas most of the amphetamines are eluted later (Nos. 8–14, t_R = 10.65–15.50 min). Because of the gradient elution, the amphetamines are eluted within a reasonable period (about 5 min) and focused as narrow peaks, which contributes to their sensitive detection.

The only drawback of the present separation is the impossibility of detecting pemoline in a complex matrix, because of its co-elution with interfering compounds (see Table I). The early elution of 4-hydroxyamphetamine does not cause a serious

TABLE I

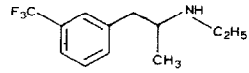
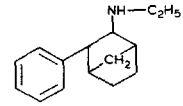
RETENTION TIMES (t_R) OF COMPOUNDS STUDIED BY GRADIENT CHROMATOGRAPHY

For conditions, see Fig. 2b; t_{Ro}, 0.60 min.

Peak No.	*Compound*	*Formula*	*t_R (min)*	*Relative standard deviation (%) (n = 6)*
1	Noradrenaline		0.60	
2	Adrenaline		0.60	
3	Dopamine		0.60	
4	5-Hydroxytryptamine		1.70	
5	3-Methyldopamine		2.60	
6	4-Hydroxyamphetamine		3.30	
7	Pemoline		3.45	1.41
8	Ephedrine		10.65	0.60
9	Amphetamine		12.45	0.69
10	Diethylpropion		12.70	
11	Mephentermin		13.35	
12	Methylphenidate		13.45	0.61

Continued on p. 64

TABLE I (*continued*)

Peak No.	*Compound*	*Formula*	t_R *(min)*	*Relative standard deviation (%) (n = 6)*
13	Fenfluramine		15.20	
14	Fencamfamin		15.50	0.61

problem as its concentration in urine is only a few percent of that of the free unchanged amphetamine[18]. In summary, the results obtained so far were considered to be satisfactory for coupling this separation with the cleaning and enrichment steps using pre-columns (see above).

Final procedure

The final experiments were performed with the set-up in Fig. 1 using the following procedure. A blank urine sample spiked with the amphetamines is introduced to the chromatograph with an all-glass 2 ml syringe through a 0.45 μm filter (Gelman Science, Ann Arbor, MI, U.S.A.). The sample is transferred by pump P from the 1 ml injection loop L1 to the PRP-1 pre-column using 5 ml of LC-grade water. Here sorption of the amphetamines and various organics occurs, whereas inorganic constituents are flushed to waste through the void channel in V4. Next, pump P is switched to deliver 5 ml of 0.001 *M* DMOA in 0.002 *M* perchloric acid. At the same time, the Aminex A-5 pre-column in V4 is switched in-line with the effluent from the PRP-1 pre-column and simultaneously valve V1 switches L1 off-line. During this step, the amphetamines are displaced from the PRP-1 pre-column by the protonated DMOA and adsorbed on the Aminex A-5 pre-column while neutral compounds are flushed to waste. The more strongly adsorbed neutral compounds remain on the PRP-1 pre-column, from which they are removed later during the cleaning steps.

After finishing the transfer, pump P is switched to deliver the weak component of the mobile phase gradient, *i.e.*, 0.01 *M* SOS–0.001 *M* perchloric acid–0.1 *M* sodium perchlorate in methanol–water (30:70, v/v). At the same time, valve V2 is switched to deliver this solvent to valve V3. After pumping 1 ml of liquid through, all connecting capillaries are filled with this solvent. Now valve V3 is switched so that this solvent enters the combination of loops L2 and L3; 0.5 ml of the weak component of the mobile phase gradient is introduced in the mixing loops L2 and L3 within 0.5 min. Valve V3 is then switched so that the mixing loops L2 and L3 are back-flushed in-line with the strong eluting component of the mobile phase delivered by a syringe pump (E, Fig. 1). Pump P is switched off; this moment is indicated by "G" in the chromatograms presented. Now the gradient of the mobile phase prepared in the mixing loops L2 and L3 is allowed to enter the analytical microcolumn packed with the reversed-phase sorbent ODS-2. At a mobile phase flow-rate of 1.2 μl/s, the weak

component of the mobile phase enters the UV detector 75 s after event "G". This moment is used for switching the Aminex A-5 pre-column in V4 in-line with the mobile phase, indicated by "I" on the chromatograms. The amphetamines are now eluted from the A-5 pre-column towards the analytical microcolumn, separated by gradient reversed-phase ion-pair chromatography and detected at 210 nm.

It should be noted that instead of the inexpensive gradient system described here, a commercially available microbore gradient pump can be used, thereby making valves V2 and V3 superfluous.

The pre-column A-5 is switched in-line with the mobile phase during the separation and is regenerated by the gradient run itself. The event "I" can also be used for the introduction of standard solutions of amphetamines directly on to the microcolumn using the void channel in V4. Of course, in this instance, the steps involving the pre-column handling are omitted while the steps necessary for gradient preparation are maintained. At event "I", pump P is switched on again to deliver 5 ml of methanol–water (50:50, v/v), 1 ml of methanol and finally 1 ml pure water. During this period (just after event "I"), valve V2 is switched so that solvents from P enter the PRP-1 pre-column, which is cleaned and reconditioned. The effluent from the PRP-1 pre-column is flushed to waste through the void channel in V4. After flushing of the PRP-1 pre-column with at least 2 ml of water, the system is ready for the introduction of the next sample. Thus, the next run is started while the actual separation is still in progress.

Applications

Human urine. Chromatograms of a fresh human urine blank and urine spiked with amphetamines obtained by the procedure described under *Final procedure* are presented in Fig. 3a and b. The reproducibility of the solute peak areas and the recoveries are summarized in Table II.

Pemoline (peak 7, see Table II) is not transferred from the sample to the separation column. This follows from its co-elution with interfering compounds (see

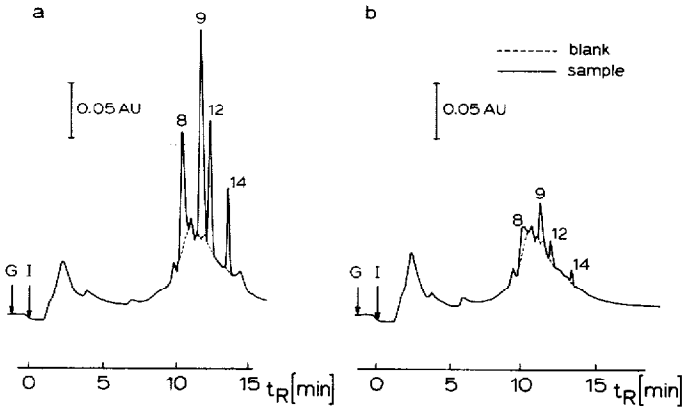


Fig. 3. Chromatograms of 1 ml of amphetamine-spiked human urine obtaned according to the final procedure described in the text. Broken lines, urine blank; solid lines, spiked urine. Numbers of compounds (see Table I) and concentrations of free base in urine (ng/ml): (a) 7, 360; 8, 830, 9, 730; 12, 740; and 14, 1070; (b) 10-fold lower concentrations. For conditions for the separation, see Figs. 1 and 2.

TABLE II

ANALYTICAL DATA FOR AMPHETAMINE SCREENING IN FRESH HUMAN URINE

Compound	*Repeatability of peak area (n = 6)*				*Recovery from urine (%)*	*Detection limit in urine (ng/ml)*
	Standards on column		*Urine with use of pre-columns*			
	Relative standard deviation (%)	*Amount injected (ng)*	*Relative standard deviation (%)*	*Amount injected (ng)*		
Pemoline	2.3	410	–	360	0	–
Ephedrine	1.6	940	5.2	830	101	100
Amphetamine	1.6	840	2.5	730	101	20
Methylphenidate	2.0	860	9.5	740	99	35
Fencamfamin	1.4	1220	12	1070	29	50

above), which are removed during enrichment and cleaning steps on the pre-columns.

The reproducibility of the peak areas is acceptable for quantifying the amphetamines below the 1 μg/ml level, when considering the manual operation of the switching valves, pumps and evaluation of the chromatograms. Even the last eluting compound of interest (fencamfamin) can be reasonably detected in spite of its low recovery. It should be noted that the detection limits in Table II are based on a comparison of peak heights with the fluctuation of the background signal caused by a blank sample. In spite of the direct injection of untreated urine, the amphetamines can be determined with a detection limit comparable to that obtained after off-line derivatization[7].

The use of a microbore column enables the sample volume to be decreased to 1 ml (*cf.* ref. 7, where 20 ml were used), which speeds up pre-column sample handling. At least 50 urine samples can be analysed without changing the pre-column. With

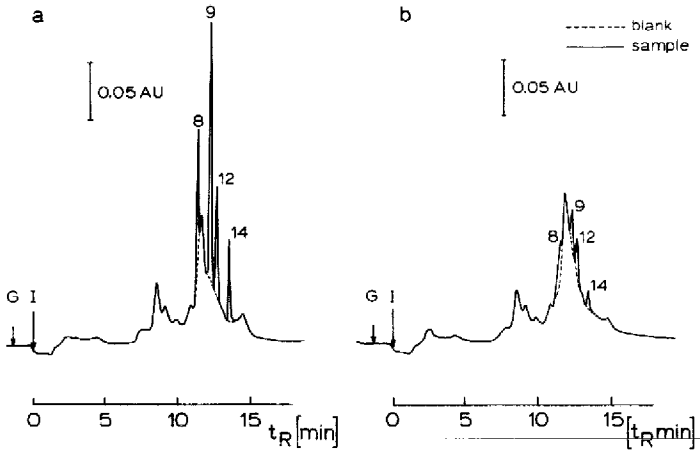


Fig. 4. Chromatograms of 1 ml amphetamine-spiked horse urine obtained according to the final procedures described in the text. Broken lines, urine blank; solid lines, spiked urine. Concentrations of amphetamines in samples: (a) see Fig. 3a; (b) see Fig. 3b. For conditions for separations, see Figs. 1 and 2.

higher numbers of analyses the decrease in the pre-column permeability becomes unacceptably high.

Horse urine. Chromatograms of spiked female horse urine and a urine blank obtained under the same conditions as for the human urine samples are presented in Fig. 4a and b. No substantial differences were found between horse male and female blank urine chromatograms. When the urine samples have been stored for a long period (2–3 weeks), the PRP-1 pre-column should be flushed with an increased amount of water (8 ml) during sample adsorption in order to decrease the high background. As with human urine, the detection limit of the amphetamines is less than 0.1 μg/ml and is determined by the background level rather than the system noise.

CONCLUSIONS

By combining the use of two sample clean-up and enrichment pre-columns in series with the period of selective solute displacement, urine samples can be screened for amphetamines without any off-line pre-treatment or derivatization. The pre-column handling follows the pattern of decreasing the sample volume after each enrichment step, so that a microbore column can be used for the final separation of the compounds of interest. A relatively cheap and non-selective sorbent can be used in the first pre-column, whereas a more expensive and more selective material is required in the second micro-pre-column. Separation of compounds with a wide range of polarity is accomplished by using gradient reversed-phase ion-pair chromatography. The drugs studied here cover the whole range of polarity of amphetamine-related drugs, so that the present procedure can be also used for screening other similar compounds. The selective pre-column enrichment allows the use of sensitive UV detection at 210 nm. The detection limits achieved are determined only by the sample background and they are not dependent on the system noise.

Sample cleaning, enrichment, mobile phase gradient preparation and solute separation have been designed to be compatible with automated liquid chromatographic procedures[8,9]. The automated screening of amphetamines in biological fluids is especially important for drug abuse and doping control when large series of samples have to be analysed within a short period.

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