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# Fast systematic approach for the determination of drugs in biological fluids by fully automated high-performance liquid chromatography with on-line solid-phase extraction and automated cartridge exchange

## Application to cebaracetam in human urine

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ABSTRACT

A fast stepwise systematic approach for the conversion of conventional reversed-phase high-performance liquid chromatographic (HPLC) assays involving liquid–liquid extraction of biological fluids into fully automated HPLC assays using solid-phase extraction and cartridge exchange is described. The suitability of this procedure is demonstrated for the determination of cebaracetam in human urine.

## INTRODUCTION

Solid-phase extraction (SPE) of drugs from biological fluids, via disposable extraction columns or by column-switching, is widely used in combination with high-performance liquid chromatography (HPLC) as an alternative to time-consuming liquid–liquid extraction (for a review see ref. 1). Recently, a fully automated system with extraction cartridge exchange (Merck OSP-2) has been introduced for the routine on-line analysis of drugs in plasma [2,3], as well as for the analysis of samples of environmental concern [4].

This paper describes a fast systematic approach to convert conventional reversed-phase HPLC methods using liquid–liquid extraction into fully automated methods using a marketed SPE device. The utility of this approach is demonstrated for the determination of a new nootropic drug, cebaracetam, 1-[4-(*p*-chlorophenyl)-2-oxopyrrolidin-1-yl-acetyl]-3-oxopiperazine, in human urine.

## EXPERIMENTAL

### *Materials and reagents*

Cebaracetam and 1-[4-(*p*-fluorophenyl)-2-oxopyrrolidin-1-yl-acetyl]-3-oxopiperazine (internal standard) were provided by Zyma (Nyon, Switzerland). Their structures are shown in Fig. 1. Internal standard and calibration solutions were prepared by dissolving the compounds in water. The reference solutions were stored at 4°C. Under these storage conditions, they were stable for three months.

All chemicals were of analytical grade from

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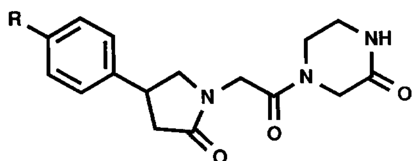



R = Cl : Cebaracetam, $C_{16}H_{18}ClN_3O_3$ ; mol. wt. 335.80
R = F : Internal standard, $C_{16}H_{18}FN_3O_3$ ; mol. wt. 319.33

Fig. 1. Structures of cebaracetam and internal standard.

Merck (Darmstadt, Germany) except anhydrous sodium dihydrogenphosphate, which was of Suprapur grade. All solvents were of HPLC grade and were also from Merck. Water was doubly distilled.

*Apparatus*

The chromatographic system used for the determination of cebaracetam after liquid–liquid extraction of urine consisted of two pumps (Model 510), an automated gradient controller (Model 680) and a WISP 710B autosampler, all from Waters Assoc. (Milford, MA, USA), and a variable-wavelength UV detector from Applied Biosystems (Model Spectroflow 783A, Foster City, CA, USA).

The chromatographic system used for the fully automated analysis has been already described [2]. Some minor modifications were made: a pump from Merck-Hitachi (Model L-6200) was used instead of two Waters Assoc. pumps, a variable-wavelength UV detector from Applied Biosystems was used instead of the fluorescence detector, and the autosampler was equipped with a 100-$\mu$l fixed loop.

Data acquisition and integration were peformed using a Maxima 820 workstation from Waters Assoc.

*Columns*

LiChroCART cartridges (4 mm × 4.0 mm I.D.; Merck), which were packed with LiChrospher 100 RP-18 (particle size 10 $\mu$m), were used for SPE. A Nucleosil 120 $C_{18}$ analytical column (125 mm × 4.0 mm I.D., particle size 5 $\mu$m) from Macherey-Nagel (Oensingen, Switzerland) was used for both conventional and automated methods.

*Sample preparation*

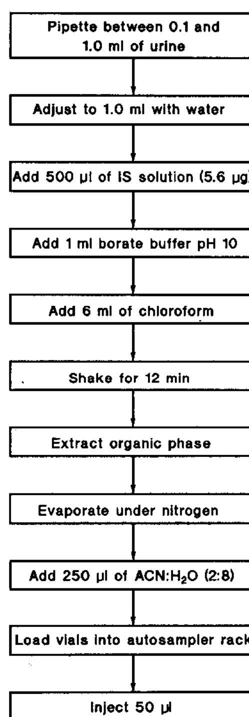
The urine samples were processed according to instructions in Fig. 2. Standard urine samples were prepared by diluting drug-free urine with reference solutions.

*HPLC conditions*

All aqueous solutions used as mobile phases were filtered on Zetapor membranes (pore size 0.22 $\mu$m; Cuno, Meriden, CN, USA). The mobile phases were quickly degassed under vacuum prior to use.

*Conventional method.* Cebaracetam extracts were analysed by isocratic elution with a mobile phase of 50 m$M$ ($Na^+$) phosphate buffer (pH 3.7)–acetonitrile (75:25, v/v). The flow-rate was 1.0 ml/min. Detection was performed at 210 nm.

*Conventional method* *SPE method*



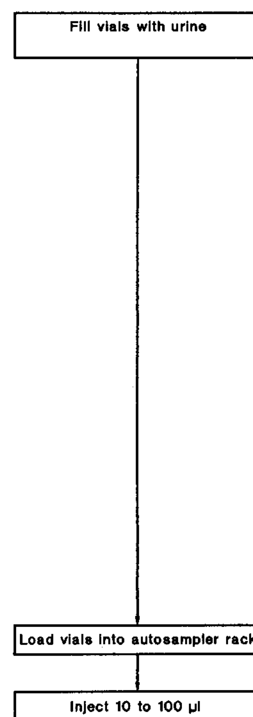




Fig. 2. Processing scheme for cebaracetam in human urine. ACN = acetonitrile.

*Automated method.* The SPE step was carried out using the experimental conditions given in Table I for the fast systematic approach. Phosphate buffer (pH 7.4) was used as mobile phase B. For the analytical separation, the elution conditions were similar to the conventional analysis. Mobile phase was 50 m*M* ($Na^+$) phosphate buffer (pH 3.7)–acetonitrile (75:25 v/v). The flow-rate was 1.0 ml/min. Detection was performed at 210 nm.

*Fast systematic approach*

The following stepwise procedure was applied to the cebaracetam assay in urine as an example of the conversion of a conventional reversed-phase HPLC method using liquid–liquid extraction into a fully automated HPLC–SPE method.

Step 1. Set suitable chromatographic parameters for the analytical separation system (column, mobile phase A, gradient or isocratic elution, detection).

Step 2. Load the OSP-2 rack with LiChroCART 4-4 cartridges packed with LiChrospher RP-18 (5 or 10 μm). Set the mobile phase for SPE: B, phosphate buffer (50 m*M*) adjusted to pH 7.4 (for basic compounds) or pH 3.0 (for acidic compounds; neutral compounds can be extracted with both mobile phases); C, acetonitrile (or methanol)–0.05% phosphoric acid (90:10, v/v). Mobile phase C was used for cartridge conditioning.

Step 3. Enter the extraction/elution/conditioning programme given in Table I in the programming device.

Step 4. Prepare reference solutions by dissolving the drug in water or in mobile phase B. Avoid the use of organic modifiers in reference solutions. Prepare spiked biological samples. Filter on single-use filter unit or centrifuge if not clear.

Step 5. Set injection volumes according to the required sensitivity. Inject the reference solution twice (the first injection should be omitted from the final analysis because of lack of cartridge conditioning), a blank and a spiked sample.

Step 6. Optimize the analytical separation, if necessary. Determine the recovery of the extraction step by comparison of the peak areas obtained by direct injection of reference solution onto the analytical column and by SPE of spiked samples.

Step 7. If the recovery is above 90% and the analytical separation fits requirements, determine the extraction cartridge capacity by injection of increasing amounts of the drug in the biological fluid. If the recovery is below 90%, omit the filtration step. If the drug is not strongly adsorbed on the filter, the extraction step should be modified. This can be optimized by varying the pH of mobile phase B (concentration or the type of buffer used), or, if necessary, by changing the RP-18 cartridges for CN or RP-8 cartridges.

Step 8. Determine the number of times that a cartridge can be used with the DIRECTION command of the OSP-2.

Step 9. Validate the method according to GLP guidelines.

*Calibration and quantification*

For the conventional method, calibration was done by linear regression of the ratio of peak heights (cebaracetam/internal standard) *versus* the given concentration ranging from 0 to 30.0 μg/ml (ISTD calibration). Linear regression of cebaracetam peak height *versus* the given concentration in about the same range was done for the automated method (ESTD calibration). Quantification was done using the calibration curves.

*Method validation*

The recoveries of the extraction step were determined by comparing the peak areas of spiked urine samples with those obtained for an equivalent amount directly injected into the analytical column. The linearity of the calibration curves was checked by their correlation coefficient in the range 0.3–30.0 μg/ml. The precision was determined in terms of repeatability and reproducibility ($n$ = 6). The intra- and inter-day accuracies were also measured ($n$ = 6). The stability of cebaracetam under the experimental conditions was determined over a 12-h period with a standard urine sample of 6.0 μg/ml. The stability of cebaracetam under the storage conditions before analysis (−25°C) was determined over four

TABLE I

EXTRACTION/ELUTION/CONDITIONING PROGRAMME OF THE FAST STEPWISE SYSTEMATIC APPROACH

| Time (min) | OSP-2 | | | | Analytical pump | SPE pump | | Comments |
|---|---|---|---|---|---|---|---|---|
| | Valve No. 1 | Valve No. 2 | Clamp | Move | Eluent A (ml/min) | Eluent B (ml/min) | Eluent C (ml/min) | |
| 0.0 | ON | OFF | ON | OFF | —[a] | 1.0 | 0.0 | Initial conditions, equilibration of both pre-column and analytical column |
| 4.5 | OFF | OFF | ON | OFF | —[a] | 1.0 | 0.0 | By-pass of extraction mobile phase |
| 4.6 | OFF | OFF | OFF | OFF | —[a] | 1.0 | 0.0 | Opening of the clamp |
| 4.7 | OFF | OFF | OFF | Pulse | —[a] | 1.0 | 0.0 | Move of the pre-column from extraction side to analytical side of the OSP-2, installation of a new pre-column at the extraction side |
| 4.8 | OFF | OFF | ON | OFF | —[a] | 1.0 | 0.0 | Closure of the clamp |
| 5.0 | ON | ON | ON | OFF | —[a] | 1.0 | 0.0 | Start of analytical separation and of data acquisition<br>Start of gradient programme if required |
| 5.1 | ON | ON | ON | OFF | —[a] | 2.0 | 0.0 | Conditioning of a new pre-column: intensive washing with aqueous mobile phase |
| 8.0 | ON | ON | ON | OFF | —[a] | 0.0 | 2.0 | Conditioning of a new pre-column; intensive washing with high content organic modifier mixture |
| 14.0 | ON | ON | ON | OFF | —[a] | 2.0 | 0.0 | Re-equilibration of the new pre-column with the aqueous mobile phase |
| 20.0 | ON | ON | ON | OFF | —[a] | 1.0 | 0.0 | Re-equilibration of the new pre-column with the aqueous mobile phase |
| 22.0 | ON | ON | ON | OFF | —[a] | 1.0 | 0.0 | Pre-column ready for a new run, waiting end of analytical separation |

[a] As set for the conventional method.

months with standard urine samples of 0.2 and 1.0 μg/ml.

## RESULTS AND DISCUSSION

The HPLC–SPE method for the determination of cebaracetam was developed by applying the fast stepwise systematic approach described.

The experimental conditions of the conventional HPLC method were set for the analytical separation (step 1). The SPE parameters were set as described, using phosphate buffer (pH 7.4) (step 2). The extraction/elution/conditioning programme was entered into the pump and event programmer (step 3). Typical chromatograms obtained for the first injection of a standard urine sample of 10.0 μg/ml, processed using both the conventional and the SPE methods, are shown in Fig. 3 (steps 4 and 5). Owing to the different HPLC systems used and to the design of the OSP-2 device, shorter retention times were observed with the automated method. No analytical separation optimization was required.

The recoveries were determined with standard urine samples of 9.0 μg/ml (step 6). Values of 85.0 ± 2.0% ($n$ = 3) were measured for the conventional method using chloroform and borate buffer (pH 10) to extract, and 92.3 ± 0.8% ($n$ = 3) for the SPE method using an octadecyl stationary phase eluted by phosphate buffer at physiological pH.

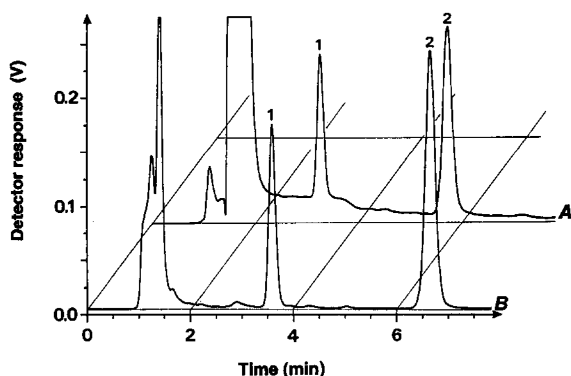



Fig. 3. Typical chromatograms of a standard urine sample of 10 μg/ml cebaracetam assayed with the SPE method (A) and the conventional method (B). Peaks: 1 = internal standard; 2 = cebaracetam. The internal standard was also added to the sample assayed with the SPE method for a better comparison of chromatograms. Injections: A, 100 μl of standard urine sample; B, 50 μl, corresponding to a urine volume of 200 μl injected directly (see Fig. 2); for the other experimental conditions, see text.

The capacity of the extraction cartridges was found to be more than 100 μg of cebaracetam (step 7). A correlation coefficient ($r$) of 0.9999 was calculated for the range 0.3–100 μg/ml. However, the capacity was actually higher, because the detector response was overloaded at *ca.* 150 μg/ml. In order to reduce the concentration range, urine samples collected in pharmacokinetic studies were either diluted with water (conventional method) or the injected volume was systematically adjusted (SPE method) for the expected concentrations. In the latter case, the peak areas obtained after injection of standards increased linearly with increasing sample size in the range 20–100 μl ($r$ = 1.0000). One cartridge could be used up to 27 times (step 8). The relative standard deviation of the cebaracetam peak height, measured after 27 successive injections of a standard urine sample of 6.0 μg/ml on the same cartridge, was 0.5%. Cartridge-to-cartridge variations and peak broadening were negligible.

Inter-assay validation data are shown in Table II (step 9). The precision and the accuracy were determined over the range 0.3–30 μg/ml. Both methods showed similar precision and accuracy. No internal standard was required for the SPE method for results similar to the conventional method. The quantitication limit was of the order of 0.3 μg/ml. Lower concentrations could be measured with less precision and accuracy. The calibration graphs relating the cebaracetam peak height to its concentration in prepared standards were linear in the range 0.3–30 μg/ml. Correlation coefficients ($r$) of 0.9998 ± 0.0001 ($n$ = 6) and 0.9999 ± 0.0001 ($n$ = 6) were measured for the conventional and the SPE method, respectively. Cebaracetam was found to be stable under experimental conditions for up to 12 h. At −25°C, no significant decrease of cebaracetam concentrations was observed for up to four months.

In our experience, most conventional reversed-phase HPLC assays involving liquid–liquid ex-

TABLE II

INTER-ASSAY PRECISION AND ACCURACY OF THE CEBARACETAM ASSAYS

| Nominal concentration (μg/ml) | Concentration found, (mean ± S.D.) (μg/ml) | Relative S.D. (%) | Confidence interval of the mean value ($P=95\%$) (μg/ml) | Accuracy[a] (%) |
|---|---|---|---|---|
| *Conventional method (n = 3)* | | | | |
| 0.32 | 0.24 ± 0.07 | 29.4 | 0.24 ± 0.17 | −24.3 |
| 4.01 | 4.03 ± 0.14 | 3.4 | 4.03 ± 0.34 | 0.4 |
| 8.02 | 8.08 ± 0.09 | 1.1 | 8.08 ± 0.22 | 0.6 |
| 16.05 | 15.85 ± 0.38 | 2.4 | 15.85 ± 0.95 | −1.2 |
| *SPE method (n = 6)* | | | | |
| 0.30 | 0.28 ± 0.04 | 15.7 | 0.28 ± 0.05 | −6.4 |
| 3.00 | 3.23 ± 0.23 | 7.1 | 3.23 ± 0.24 | 7.6 |
| 9.00 | 9.08 ± 0.69 | 7.6 | 9.08 ± 0.72 | 0.9 |
| 30.00 | 29.96 ± 1.12 | 3.7 | 29.96 ± 1.18 | −0.1 |

[a] Defined as the percentage deviation between the mean concentration found and the theoretical concentration.

traction using hydrocarbons, chlorinated solvents, ethers or ethyl acetate can be converted into HPLC–SPE automated assays with the stepwise procedure described above and the OSP-2 on-line sample preparator. The procedure has been applied with success to the determination of several drugs and metabolites in plasma, serum or urine in our laboratory [5]. In order to decrease sample viscosity, plasma and serum were diluted 1:1 with mobile phase B prior to injection.

CONCLUSION

The fast systematic approach described is suitable for the conversion of conventional reversed-phase HPLC assays involving liquid–liquid extraction into fully automated HPLC–SPE assays using an on-line preparator Merck OSP-2. Its application to the determination of cebaracetam in human urine demonstrates its utility.

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