Liquid Chromatographic Sample Cleanup Coupled On-Line with Gas Chromatography in the Analysis of Beta-Blockers in Human Serum and Urine

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Abstract

An on-line coupled reversed-phase liquid chromatographic–gas chromatographic (LC–GC) method with minimal manual sample preparation is developed for the analysis of metoprolol, oxprenolol, propranolol, timolol, and codeine (as an internal standard) in human serum and urine. The method is based on a loop-type interface and concurrent eluent evaporation technique. On-line liquid–liquid extraction (LLE) is used to extract the analytes from aqueous eluent to organic solvent before injection onto the GC, and the two phases are separated with a sandwich-type phase separator. The LC is used for cleanup, and the GC is used for the final separation and detection of the analytes. Total analysis time is less than 45 min, which is much less than those of traditional analysis methods. Recoveries in LC cleanup and on-line LLE are excellent. A marked increase in the recoveries with on-line LLE is obtained by heating the aqueous eluent and the extraction coil. Linearity and repeatability of the method are good for both serum and urine, and the limits of quantitation for the analytes are 18–44 ng/mL.

Introduction

Demand continues to grow for the analysis of bodily fluids for drugs. The most common methods in drug analysis are chromatographic ones that provide efficient separation with sensitive detection of the analytes in complex sample matrices. Usually the drugs are analyzed by gas chromatography–mass spectrometry (GC–MS) (1–3), but high-performance liquid chromatography (HPLC) (4), thin-layer chromatography (TLC) (5), capillary electrophoresis (CE) (6–7), and to a lesser extent supercritical fluid chromatography (SFC) (8) have also been used. Though sample preparation is almost always required in these techniques, the importance of this step is not always appreciated.

There are several objectives in sample preparation for the bioanalysis of drugs. First is the cleanup of the sample by selective removal of all endo- and exogenous compounds in the matrix that could plug the chromatographic column or interfere with the assay or separation. Because a common pathway in drug metabolism is conjugation with glucuronic acid, glutathione, sulphate, or cleavage with proteins, the analytes must also be released from the conjugates when the total concentration of the parent drugs is to be determined. Conjugates can be cleaved specifically through the use of enzymes or nonspecifically through acidic or basic hydrolysis. Another step in the sample preparation is the reconcentration of the sample. In addition, it is often necessary to derivatize the sample before the analysis in order to improve detectability, increase volatility, or decrease the adsorptivity or reactivity of the analytes.

In the bioanalytical methods presently employed, the sample preparation is usually done by liquid–liquid extraction (LLE) or solid-phase extraction (SPE). A major advantage of LLE is selectivity; depending on the choice of the solvent and pH, analytes can be extracted from most of the endogenous components. The recovery can be enhanced by successive extractions, a large excess of extracting solvent, or by salting-out techniques. Therefore, the LLE technique is highly versatile and well-documented. SPE methods have been widely employed in the preparation of biological samples. Compared with LLE, SPE is simpler, faster, and requires much less solvent. However, both LLE and SPE are tedious, labor-intensive, and often even imprecise.

In recent years, considerable attention has been given to the development of on-line sample preparation methods such as column switching techniques in LC (4,9), on-line dialysis (10), and hyphenated chromatographic techniques such as SPE–LC (11) and LC–GC (12–14). Automated sample handling procedures not only shorten the total time of analysis but also usually provide better accuracy and precision than manual techniques. Also, in off-line methods, usually only a fraction of the sample is injected into the chromatographic instrument after tedious sample preparation. In contrast, in on-line methods, most of the sample material can be utilized in the final analysis, and sensitivity is enhanced.

Through coupling of LC and GC, the resolving power and large sample capacity of LC can be exploited in the cleanup and

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concentration of the sample, and the high separation efficiency and sensitivity of GC can be used in the final analysis of the compounds. Coupling reversed-phase LC and GC requires special techniques because the introduction of large volumes of aqueous eluent to the GC column is liable to cause serious problems. Usually the aqueous eluent is changed to a suitable organic solvent before the GC analysis. One method of doing this is on-line LLE. In on-line LLE, the aqueous eluent is changed to organic solvent by continuous extraction, and the two phases are then separated in the phase separator. On-line LLE is especially suitable for the analysis of complex sample matrices because it also works as an additional cleanup step.

The aim of this work was to develop a reversed-phase LC–GC method requiring minimal sample preparation for beta-blockers in human serum and urine with codeine as an internal standard. The method was based on on-line LLE, a loop-type interface, and on-line derivatization. Efficiencies of the LC cleanup, LLE, and derivatization procedures were investigated. Also, the effect of temperature on on-line LLE was studied. The linearity and repeatability of the method were studied as well as quantitation limits for the analyte. Furthermore, the on-line derivatization was compared with existing off-line methods.

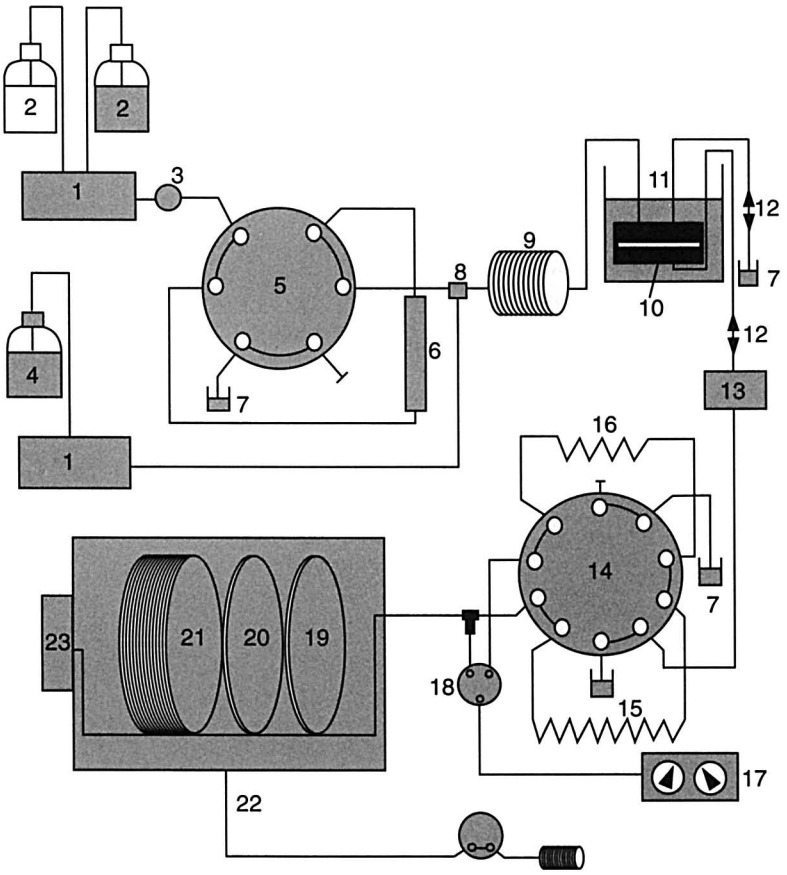

Figure 1. Schematic drawing of the on-line coupled reversed-phase LC–GC system. 1, pump; 2, eluent; 3, injector; 4, extraction solvent; 5, six-port valve; 6, column; 7, waste; 8, T-piece; 9, extraction coil; 10, phase separator; 11, ice bath; 12, restrictor; 13, diode-array detector; 14, 10-port valve; 15, sample loop; 16, reagent loop; 17, helium; 18, three-port valve; 19, retention gap; 20, retaining precolumn; 21, separation column; 22, solvent vapor exit; 23, flame-ionization detector.

Experimental

Materials

All solvents were HPLC-grade. Acetonitrile, methanol, dichloromethane, boric acid, ethyl acetate, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Metoprolol tartrate, oxprenolol hydrochloride, propranolol hydrochloride, and timolol maleate were obtained from Sigma (St. Louis, MO). Codeine phosphate was purchased from YA (Helsinki, Finland), *N,O*-Bistrimethylsilylacetamide (BSA) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) were from Sigma, and *N,O*-bistrimethylsilyl-trifluoroacetamide (BSTFA), *N*-methyl-bis(trifluoroacetamide) (MBTFA), and trifluoroacetic anhydride (TFAA) were from Merck. Betaglucuronidase enzyme (*Helix pomatia*) was from Biosepta (Villeneuve-la-Garenne, France).

Instrumentation and conditions

The coupled LC–GC system is shown in Figure 1. The LC was a Hewlett-Packard (Amsterdam, The Netherlands) 1090 with a

Table I. Analysis Procedure

Analysis step	Procedure
Injection	100 µL sample volume is injected into the LC column.
Washing step	LC column is washed with boric acid for 3–5 min.
Elution step	Six-port valve is switched and analytes are eluted to the extraction coil with boric acid–acetonitrile as the eluent (73:27, 78:22, v/v).
On-line LLE	Heated LC eluent and CH_2Cl_2 are mixed in T-piece, and analytes are extracted into organic solvent in the heated extraction coil.
Phase separation	Aqueous eluent and CH_2Cl_2 are separated in the cooled sandwich-type phase separator. Aqueous phase goes to waste, and organic solvent goes through DAD to the sample loop in the 10-port valve.
Transfer	When the fraction containing the analytes, controlled by DAD, is in the sample loop, the 10-port valve is switched, and the carrier gas pushes the content of the sample loop followed by derivatization reagent, into the GC.
On-line derivatization	SVE is open during the eluent evaporation and is closed after that. Derivatization takes place after the evaporation and the GC analysis start.

diode-array detector (DAD). The volume of the injector loop was 100 µL. The six-port valve for backflushing the LC column was from Rheodyne (Cotati, CA). Organic solvent was delivered by a Jasco (Tokyo, Japan) pump. The extraction coil material was PEEK (3 m × 0.75-mm i.d.), and the other tubing was stainless steel. The sandwich-type phase separator (15) was made in our laboratory. The LC–GC interface consisted of a 10-port Rheodyne valve equipped with a 940-µL sample loop and an additional 78-µL loop for the derivatization reagent. The GC was a Carlo Erba (Milan, Italy) Mega series 5300 equipped with a flame ionization detector.

LC separations were performed on a 20- × 2.1-mm-i.d. column dry-packed with Capcell Pak C_{18} SG-120, 5-µm particle size (Shiseido, Japan). The LC mobile phases were 0.05M boric acid adjusted to pH 10.2 with NaOH (washing step) and the same buffer with 27 or 22% acetonitrile (elution step). Mobile phases were filtered and degassed before use. The flow rate of the mobile phase was 0.8 mL/min. Diode-array detection was at 240, 254, and 280 nm to control the cutting of sample fraction.

Columns used in GC separation were a 3-m × 3.2-mm-i.d. fused-silica DPTDMS deactivated retention gap (BGB Analytic AG, Zurich, Switzerland), a 3-m × 0.32-mm-i.d. fused-silica retaining precolumn coated with a 0.25-µm film of BGB-5 (BGB Analytik AG), and a 12-m × 0.32-mm-i.d. fused-silica capillary column coated with a 0.1-µm film of BGB-5 (BGB Analytik AG); all were connected via press fit connectors. The oven temperature was held at 92°C for 12 min for concurrent evaporation of the eluent and then increased to 120°C at 15°C/min, to 220°C at 3.5°C/min, and to 280°C at 15°C/min. The detector temperature was 300°C. The carrier gas was helium, and the flow rate was 15.5 mL/min. The analysis procedure is given in Table I.

Table II. Effect of Temperature on On-Line LLE*

	Extraction yields					
	23°C	30°C	40°C	45°C	50°C	55°C
Metoprolol	1.00	1.45	1.62	1.93	2.26	1.62
Oxprenolol	1.00	1.50	1.89	3.98	2.56	1.76
Propranolol	1.00	1.40	1.53	2.14	1.75	1.53
Timolol	1.00	1.39	1.48	2.26	2.73	2.19
Codeine	1.00	1.70	1.83	2.39	2.12	1.64

* Calculated with respect to extraction yields at ambient temperature from six replicate runs.

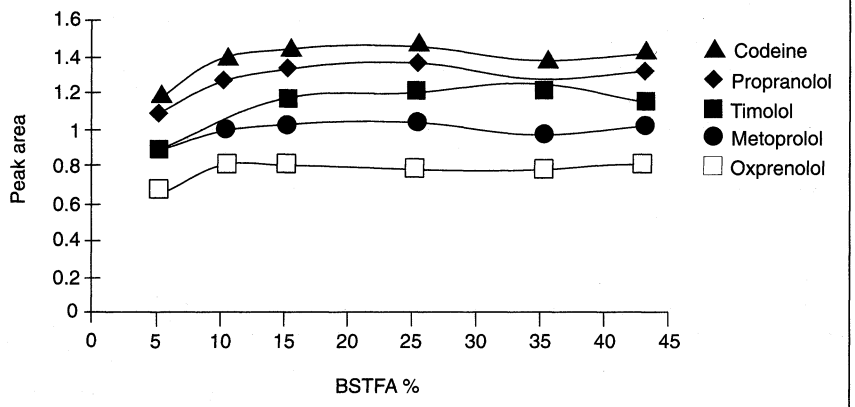

Figure 2. Effect of BSTFA concentration in CH_2Cl_2 to the peak areas of the analytes. Concentration of the analytes was 1.25 µg/mL.

Off-line derivatization methods

The analytes were manually extracted from boric acid buffer solution into CH_2Cl_2 before derivatization to give a concentration of 1.25 µg/mL. After derivatization, the diluted samples (at 1.25 µg/mL) were injected into the 940-µL sample loop in the 10-port valve, the reagent loop was filled with CH_2Cl_2, and the contents of the two loops were injected into the GC as in the LC–GC procedure (the oven temperature was 92°C, and the flow rate of helium was 15.5 mL/min). For the off-line derivatization studies, the GC was taken apart from the LC.

In method A, the sample was evaporated to dryness, 50 µL BSTFA was added to the residue, and the mixture was heated at 60°C for 30 min and diluted with CH_2Cl_2 before injection.

In method B, the sample was evaporated to 100 µL, after which 100 µL MSTFA was added to the residue, the mixture was heated at 60°C for 5 min, and 30 µL MBTFA was added. The mixture was then heated again at 60°C for 5 min and diluted with CH_2Cl_2 before injection.

In method C, the sample was evaporated to dryness, and 50 µL TFAA–ethylacetate was added to the residue; the mixture was heated at 60°C for 40 min, evaporated to dryness, and diluted with CH_2Cl_2 before injection.

Samples

Standard solutions of the drugs were made by dissolving an appropriate amount of each drug in methanol to give a concentration of 1 mg/mL. The solutions were stored at 4°C. Spiked samples were prepared by adding standard solutions to drug-free urine or serum. Drug-free urine was collected from healthy volunteers after a 2-week caffeine-free diet, and dried control serum was diluted with distilled and deionized water. Authentic samples were collected from healthy volunteers 4 h after the administration of propranolol. The dose of propranol was 10 mg.

Urine samples were diluted (1:1, v/v) with 0.05M boric acid (pH 10.2) and filtered. Serum samples were diluted (1:1, v/v) with water, and phosphoric acid (8%) and carboxymethylcellulose (CMC) (26 mg/mL) were added to the sample before filtration with 0.45-µm filters (Gelman Sciences, Ann Arbor, MI). The samples were hydrolyzed with *Helix pomatia* to cleave the glucuronic conjugates (1 mg per 1 mL of urine) at 37°C for 45 min before addition of the standard and dilution.

Results and Discussion

Many parameters in the coupled reversed-phase LC–GC system must be taken into consideration when optimizing the method. Not only must the LC and GC parameters be

carefully chosen, but the interface must be well-designed. Of the several approaches available for combining LC and GC, the loop-type interface are ideal for the relatively polar and non-volatile drugs such as beta-blockers (13). The design and use of this interface are simple; the only parameter that has to be optimized is the transfer temperature. An additional feature is that on-line derivatization can be used with this interface. Because of the problems that arise when large volumes of aqueous eluents are injected into GC columns, it is advisable to replace the reversed-phase eluent with organic solvent before GC analysis by using a trapping column, SPE, or on-line LLE. For complex samples, on-line LLE offers the additional advantage of also serving as a cleanup step.

The evaporation of large fractions (greater than 200 µL) of liquid is best done by concurrent eluent evaporation technique (CCEE) (16). In CCEE, the eluent is completely evaporated during its introduction to the GC, and because practically no liquid floods to the column, there is no need for long retention gaps, which could restrict the sample volume. Because there is no flooded zone, however, there is also no solvent trapping of the volatile compounds, which means that the method is limited to analytes with intermediate to high elution temperatures. Solvent trapping can nevertheless be achieved by adding a small amount of high-boiling cosolvent to the main solvent, which will spread to the precolumn and provide conditions for solvent trapping.

For beta-blockers, which have intermediate elution temperatures, we used CCEE during the transfer of sample fractions to the GC, and the temperature of the transfer was optimized to achieve a fast evaporation of the solvent together with efficient derivatization of the analytes. An early vapor exit was used to remove the excess solvent and also to enhance the speed of evaporation. We have described the setup earlier in more detail (13).

LC cleanup

Direct injection of serum to the LC column is more problematic than direct injection of urine because serum proteins tend to adsorb on the column packing material and clog the column. This problem can be avoided by using a polymeric stationary phase (3,17) or adjusting the eluent composition (3,18). Unfortunately, in LC–GC coupling, any change in eluent composition will affect the on-line LLE because the pH of the eluent must be optimum to achieve maximum LLE efficiency. Micellar eluents cannot be used either because that would disturb the GC separation. We decided to avoid protein adsorption by changing the composition of the serum sample itself by adding phosphoric acid and CMC before the injection (19–21).

In our LC method for sample cleanup, the sample was injected to the LC column with boric acid eluent. A short 2-cm column was used to avoid separation between the analytes and get as little fractioning as possible for the GC analysis. The column was washed with the buffer to elute the interfering matrix compounds to waste, and the analytes of interest were retained by the stationary phase. The LC programs for urine and serum samples were slightly different. A 3-min washing period was sufficient to remove the endogeneous compounds from urine samples, but a longer washing time (5 min) was required to remove the proteins and other matrix compounds from the serum. Moreover, as noted above, phosphoric acid and CMC were added to the serum sample before the injection to avoid adsorption of proteins on the column packing material (19–21). These compounds do not react with the sample itself, but they protect the particles of packing material from the pro-

Table III. Derivatization Efficiencies of Three Off-Line Derivatization Methods and the On-Line Method*

	A	B	C	On-line
Metoprolol	0.76	1.16	0.54	1.0
Oxprenolol	1.02	0.43	0.58	1.0
Propranolol	0.82	1.38	0.54	1.0
Timolol	0.92	1.66	–	1.0
Codeine	1.18	1.52	–	1.0

* Calculated with respect to the on-line method from six replicate runs.

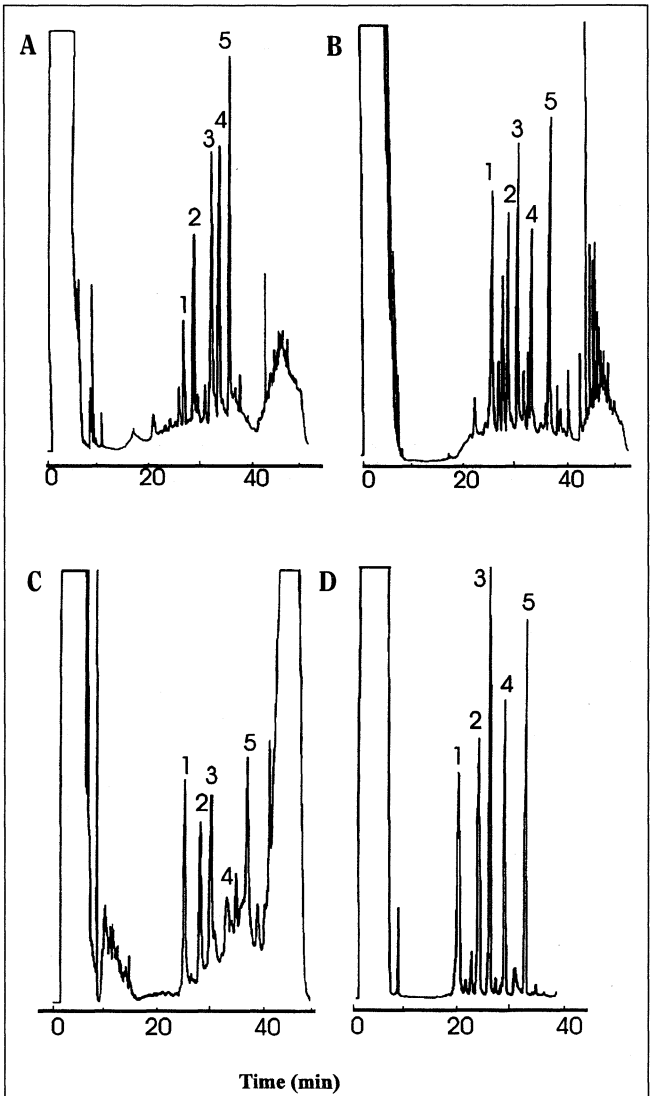

Figure 3. GC chromatograms of the analytes derivatized (A) off-line with method A, (B) off-line with method B, (C) off-line with method C, and (D) with the on-line method. Peaks: 1, oxprenolol; 2, metoprolol; 3, propranolol; 4, timolol; and 5, codeine.

tein adsorption. The CMC covers the free silanol groups, and it has been assumed that the phosphate can form complexes with silanol groups that decrease the adsorption of proteins and peptides (20,21). After the washing step, the six-port valve was switched, the eluent composition was changed, and the analytes were eluted in backflush mode to the extraction coil. No sample loss was noticed during the washing step. After approximately 180 injections of urine or 120 injections of serum, it was necessary to replace the first 2–4 mm of the column packing material.

On-line LLE

In the extraction of the analyte from aqueous (α) to organic phase (β), an equilibrium distribution is established between the two phases, which follows the equation

$$(c_i^{\beta}/c_i^{\alpha})_{eq} = \exp(-\Delta\mu_i^0/RT) \qquad \text{Eq 1}$$

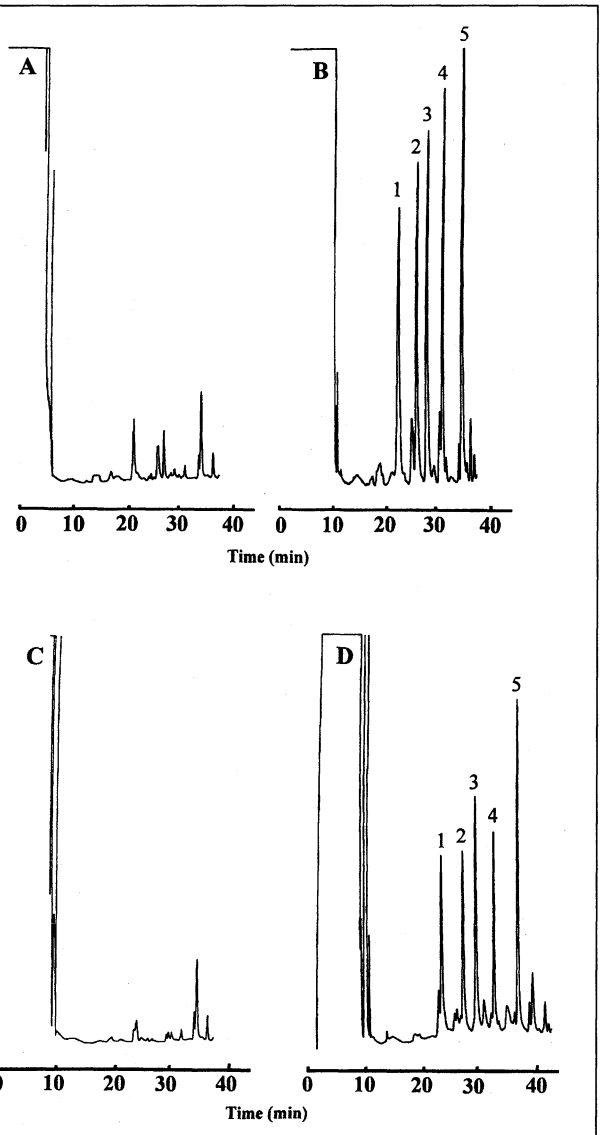

Figure 4. GC chromatograms of (A) blank urine, (B) beta-blockers in urine, (C) blank serum, and (D) beta-blockers in serum. Concentration of the analytes was 4 µg/mL. Peaks: 1, oxprenolol; 2, metoprolol; 3, propranolol; 4, timolol; and 5, codeine.

where c_i^{α} and c_i^{β} are the concentration of analyte *i* in the two phases, *R* is the gas constant, *T* is the temperature, and $\Delta\mu_i^0$ is the difference in the standard chemical potential of the analyte in the two phases (22).

The choice of organic solvent, pH of the aqueous phase, and ratio of the two phases affect $\Delta\mu_i^0$ and therefore the distribution ratio. Through the careful choice of these conditions, the distribution ratio can be maximized.

Even though the equilibrium state is not fully reached in the on-line extraction, the equation is still valid and can be used in the optimization. In the LC–GC coupling, the system itself restricts the choice of extraction solvent and the pH and composition of the aqueous eluent in some respects. We chose dichloromethane for the extraction solvent. Besides having a high extraction potential for the beta-blockers, it was easily separated in the phase separator and, because of its low boiling point, suitable for GC analysis. Boric acid (0.05M) at pH 10.2 was used as the aqueous buffer. There are also other parameters in the on-line LLE that had to be taken into account in optimizing the extraction efficiency (e.g., material and dimensions of the extraction tube, flow rate and flow ratio of the two phases, and the design of the phase separator) (23).

As can be seen from Equation 1, a more straightforward way to influence the extraction ratio is to change the temperature. We studied the effect of temperature on the LLE by heating the extraction coil and the LC eluent. Because the pressure inside the extraction tubing was far above the ambient pressure, it was possible to increase the temperature above the boiling point of the dichloromethane (39°C at 1 bar). Increasing the temperature by 20°C enhanced the extraction recovery between 1.8- to 3.2-fold, depending on the analyte (Table II). At 45°C, band broadening disturbed the collection of the sample fraction. The absolute recoveries in on-line LLE at 45°C were 104% for metoprolol, 86% for oxprenolol, 70% for propranolol, and 103% for codeine. The absolute recovery of timolol could not be determined because recrystallization of the compound in free form was not successful.

Derivatization

The adsorptivity and reactivity of many pharmaceuticals make their GC separation difficult without derivatization. The derivatization of analytes should meet the following requirements: the reaction should be fast, quantitative, and repeatable, and the derivatives should be stable enough for GC separation. Many of the common derivatization reactions are affected by moisture or oxygen or the derivatives are susceptible to degradation, but these problems are minimized or completely eliminated if the derivatization is carried out on-line in a closed system. For on-line derivatization, the reagent should be of high purity and volatility, and the by-products formed in the reaction should be highly volatile.

The three most common derivatization methods for beta-blockers are acetylation, silylation, and cyclization (24). Of these three, trimethylsilylation is the most suitable for on-line procedures. Acetylation is a much slower reaction, and excess reagent will affect the separation. In cyclization, the boronic acids that form during the derivatization reaction will disturb the separation and detectability of the analytes. From the wide

variety of silylation reagents, only BSA, BSTFA, and MSTFA have been used in the quantitative analysis of beta-blockers. There were no significant differences in the derivatization efficiencies of these reagents, but BSTFA gave the lowest background and was chosen accordingly.

In the on-line procedure, the derivatization reagent was transferred to the GC column immediately after the sample fraction. It can be assumed that the reagent reached the sample components in the retention gap when the evaporation of solvent was nearly complete. If the reagent was transferred to the column before the sample fraction, the derivatization was not satisfactory, and the peaks were broad and split. The reagent was used in excess, which caused some problems with the detector due to silica deposits. This could easily be avoided, however, by optimizing the concentration of the reagent diluted with dichloromethane (Figure 2). To evaluate the efficiency of the on-line derivatization, three off-line derivatization methods were tested, and the results were compared (Table III). The derivatization with off-line acetylation was not satisfactory; the derivatization yield was poor compared with the other methods, and timolol and codeine could not be separated from the reagent background (Figure 3). Off-line silylation and silylation combined with acetylation gave better results, but there were still many extra peaks due to the excess reagent (Figures 3B and 3C). As shown in Figure 3D, the on-line derivatization provided a much better chromatogram with fewer interfering peaks than the off-line methods, and the derivatization yield was comparable with the off-line methods. The good results of the on-line derivatization procedure were the results of careful optimization of the conditions, which is not possible in off-line procedures. Furthermore, the reaction was carried out in helium atmosphere and in high pressure, which probably enhanced the derivatization reaction.

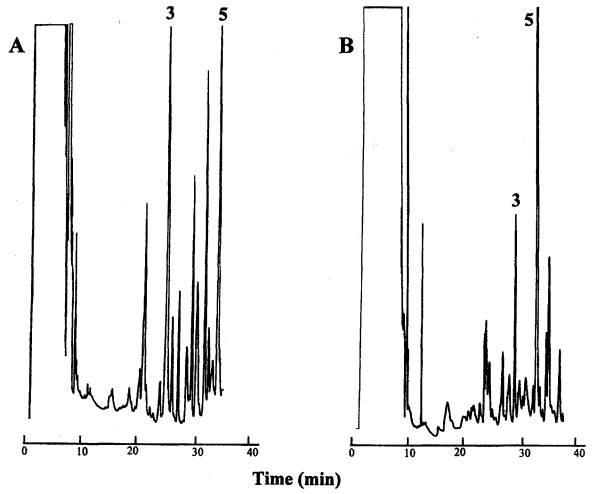

Figure 5. GC chromatograms of patient samples of (A) urine and (B) serum after a dose of propranolol. Samples were collected 4 h after the 10-mg dose was administered. Propranolol was found at 1.46 µg/mL in urine and at 0.50 µg/mL in serum. Peaks: 1, oxprenolol; 2, metoprolol; 3, propranolol; 4, timolol; and 5, codeine.

Linearity, repeatability, and sensitivity

To evaluate the applicability of the method to the quantitative determination of the beta-blockers, we investigated the linearity, repeatability, and precision of the method and the quantitation limits for the analytes.

Human serum and urine contain various compounds that may interfere with the separation of the analytes. Our on-line cleanup procedure was effective, however, and most of the interfering components were eliminated (Figure 4). Figure 5 shows GC chromatograms of authentic patient urine (A) and serum (B) samples collected 4 h after the administration of propranolol (10 mg). As can be seen from the chromatograms, a higher concentration of propranolol was found in urine than in serum.

Table IV gives the linearities, precision, and repeatability of the method and the quantitation limits for the analytes in serum and urine. The linearities were investigated in the concentration range 0.1–16 µg/mL, and they were good for all the analytes in both serum (0.956–0.997) and urine (0.983–0.986). Repeatabilities (5.6–14.4%) were also good. The repeatabilities of relative retention times, which were calculated against the internal standard, codeine, were excellent (less than 0.39% for all beta-blockers). Quantitation limits were better in urine (0.018–0.026 µg/mL) because, to avoid precipitation of the serum proteins to the LC column, the eluent and the extraction coil were not heated during the serum analysis.

Table IV. Linearities, Quantitation Limits, and Repeatabilities of Relative Retention Times and Peak Areas Calculated from Five Replicate Runs

		Metoprolol	Oxprenolol	Propranolol	Timolol	Codeine
Linearity*	Urine	0.985	0.986	0.983	0.985	0.983
	Serum	0.956	0.987	0.996	0.997	0.997
Quantitation limit (µg/mL)†	Urine	0.020	0.026	0.022	0.018	–
	Serum	0.039	0.040	0.044	0.037	–
Repeatability‡ (%RSD)	t_{rel}	0.11	0.39	0.13	0.05	–
	Area	9.9	5.7	6.9	6.8	14.4

* Concentration: 0.1, 0.5, 2, 4, 12, and 16 µg/mL.
† Signal-to-noise ratio = 4.
‡ Concentration: 4 µg/mL.

Conclusion

The reversed-phase LC–GC method allowed the separation of beta-blockers in human urine and serum with minimal manual sample pretreatment. At 45 min, the total analysis time was much shorter than in traditional methods. The cleanup procedure with LC preseparation combined with on-line LLE was efficient and allowed removal of most of the endogeneous matrix compounds. Heating the extraction coil and the LC eluent improved the extraction efficiency of the on-line LLE dramatically. Use of a loop-type

interface and CCEE techniques during the transfer of the sample to the GC made it possible to inject large sample fractions and thus increase the sensitivity. Also, with loop-type interface, it was easy to use on-line derivatization of the analytes. Furthermore, the on-line derivatization was successful, and the yield was comparable to results obtained with traditional off-line methods. In addition, the on-line derivatization provided a much better chromatogram with fewer interfering peaks than the off-line methods. The reversed-phase LC–GC method proved to be linear, repeatable, and sensitive and thus applicable to the quantitative and reliable analysis of drugs in biological fluids.

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