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Enhancement of detection sensitivity and cleanup selectivity for tobramycin through pre-column derivatization

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ABSTRACT

Reversed-phase high-performance liquid chromatography, UV detection and reversed-phase solid-phase extraction (SPE) as analytical methods for pharmaceutical compounds face a challenge when the compound is rather polar and lack UV absorptivity. A good example is tobramycin. To overcome these problems, a method has been developed using pre-column derivatization of tobramycin with *o*-phthalaldehyde and automated with an autosampler with microrobotic routines.

The detection enhancement of the derivatives was achieved by using fluorescence detection which was forty times more sensitive than using UV detection.

Recovery studies of standards and spiked serum samples show that pre-SPE derivatization significantly enhances the recoveries (by at least a factor of 3) and the quality of cleanup over post-SPE derivatization.

INTRODUCTION

The combination of reversed-phase high-performance liquid chromatography (HPLC) and UV detection is among the most common methods of analysis for pharmaceutical compounds. This analytical methodology has practical limitations when the analyte (1) has low UV absorptivity, (2) absorbs only in the low UV or (3) is rather polar. Low UV absorptivity causes low detection sensitivity and unacceptable detection limits. Detection at the lower UV wavelengths is more prone to interferences and instability of the chromatographic baseline. High polarity decreases reversed-phase retention which increases the potential for coelution of polar matrix components from a sample such as serum or plasma. Furthermore, this loss of selectivity limits the quality of sample cleanup by reversed-phase solid-phase extraction (SPE).

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Tobramycin, an aminoglycoside antibiotic, is a good example of a pharmaceutical that carries all three limitations. The ratio of amino and hydroxyl groups to the hydrocarbon backbone yields a relatively polar molecule. The lack of unsaturation limits its UV absorbance detection to low UV wavelengths and poor UV absorptivity.

Post-column derivatization of tobramycin has been performed using *o*-phthalaldehyde (OPA) [1–2]. The pumps, tees and mixers required by post-column derivatization increase both the complexity and cost of the HPLC system. The post-column reaction enhances detection sensitivity and detection selectivity *versus* coeluting solutes that are not derivatized by OPA. It does not, however, alter the mechanism of the separation. Consequently, a polar analyte like tobramycin may not be adequately separated from matrix components. Longer run times (slower separations) may be required to minimize this limitation.

Pre-column derivatization of tobramycin has also been performed. 1-Fluoro-2,4-dinitrobenzene

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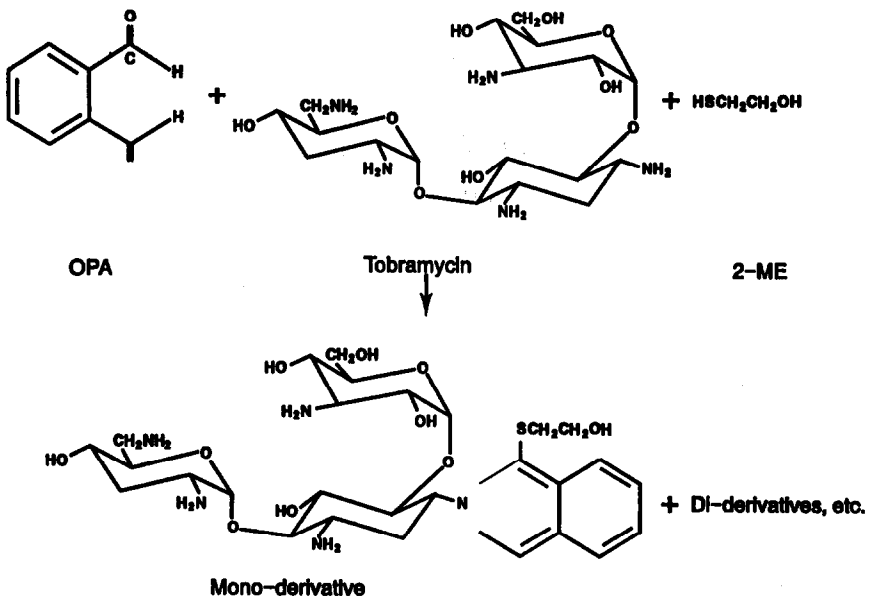

Fig. 1. Analysis of tobramycin using pre-column derivatization (OPA).

(FDNB) was used as the derivatization reagent [3,4]. The major drawback of this method is the toxicity of the reagent. The reaction is also slow and requires heating. OPA has been commomly applied to pre-column derivatization of primary amino acids [5–7]. Derivatization of aminoglycoside antibiotics in serum or plasma samples using OPA and extraction of the derivatives with ethyl acetate have been reported [8,9]. Essers [10] designed an automated HPLC method for the determination of aminoglycosides in serum using a column-switching technique for pre-column sample cleanup and derivatization.

In this study, the derivatization of tobramycin with OPA (Fig. 1) is automated using an LC autosampler from Varian that has an AutoMix microrobotic feature. AutoMix permits all sample preparation steps, *i.e.*, reagent addition, mixing and extraction, to be carried out automatically [11].

Due to multiple amino sites on the structure, it is conceivable that more than one derivative may be obtained. The derivatization of individual amino groups should be affected by the reaction time, reaction medium and steric hindrance.

In *Part 1*, tobramycin is derivatized, separated and detected by fluorescence and by UV simultaneously. The results are compared. In *Part 2*, the effect (on recovery) of derivatization before and after SPE of serum samples is compared. When reversed-phase SPE is used in serum sample cleanup, the polarity of a compound like tobramycin should cause poor selectivity and low recovery in the cleanup. Derivatization is performed to render the analyte more non-polar, thus enhancing its retention on the reversed-phase SPE column relative to the non-derivatized polar constituents in a serum matrix. In *Part 3*, linearity and reproducibility for standards are determined. In *Part 4*, recoveries and reproducibility for spiked serum samples are studied in the clinical range.

EXPERIMENTAL

Instrumentation

The HPLC system, consisting of 9010 pump, 9095 AutoSampler, Fluorichrom II fluorescence detector, LC Star Workstation and MicroPak SP C_8 15 cm × 4 mm column, was from Varian Chromatography Systems (Walnut Creek, CA, USA).

Materials

Tobramycin standard (Lot No. Y07147) was a

gift from Eli Lilly(Indianapolis, IN, USA). Potassium hydroxide, 45%, analyzed-reagent grade and potassium phosphate, monobasic, analyzed-reagent grade were from J. T. Baker (Phillipsburg, NJ, USA). Boric acid, analytical-Reagent grade was from Mallinckrodt (St. Louis, MO, USA). 2-Mercaptoethanol (2-ME) was from Pierce (Rockford, IL, USA). OPA was from Pickering Lab., (Mountain View, CA, USA). Acetic acid, glacial, acetonitrile, HPLC grade, acetone, HPLC grade, and methanol, HPLC grade were from Burdick & Jackson (Muskegon, MI, USA). 5-Sulfosalicylic acid was from Sigma (St. Louis, MO, USA). Blank serum (freeze dried) was from Utak Labs., (Saugus, CA, USA). Bond Elut C_{18} cartridges were from Varian Sample Prep Products (Harbor City, CA, USA).

Methods

OPA reagent was prepared as in Bäck *et al.*'s report [8]: 1 g of boric acid is dissolved in 38 ml of deionized water and adjusted to pH 10.4 with potassium hydroxide (450 g/l). In another vessel, 200 mg of OPA are dissolved in 2 ml of methanol and 400 μl of 2-mercaptoethanol are added. The two solutions are mixed and stored under nitrogen at +4°C.

Part 1: Derivatization of tobramycin standard and linearity study. Tobramycin stock standard (1.28 n*M*) was prepared by dissolving 30 mg in water. Working standards were prepared by dilution of the stock standard in water–acetonitrile (20:80) to 128, 96, 64, 32, 16 and 8 μM. A blank without tobramycin was also included for comparison.

The 9095 AutoSampler was programmed to AutoMix 40 μl of tobramycin standard (8–128 μM) and 40 μl of OPA reagent, stand for the reaction time (0–30 min), and finally inject 25 μl onto the HPLC System. Simultaneous detection with UV 254 nm and fluorescence (excitation 340 nm, emission 450 nm) was used for comparison.

HPLC conditions are shown in Table I.

Part 2: Comparison of recovery of tobramycin derivatized after solid phase extraction (method I) and before solid phase extraction (method II). Working standards (640 and 480 μM) were prepared by dilution of stock standard with water.

Spiked serum samples were prepared by reconstituting serum with the working standards. A blank was prepared by reconstituting with water.

For method I, the 480 μM standard and spiked serum samples were used. A 500-μl aliquot of each was deproteinated[a] with 500 μl of 10% (w/v) sulfosalicylic acid, vortexed and centrifuged. For method II, the 640 μM standard and spiked serum sample were used. A 100-μl aliquot of each was diluted 5 times[b] with water (final concentration: 128 μM) and deproteinated as in method I.

The rest of the procedure is shown in Table II. Bond Elut C_{18} cartridges were used for SPE.

HPLC conditions are shown in Table I.

Part 3: Linearity and reproducibility of standards using method II. Working standards (128, 96, 64, 32 and 12.8 μM) were prepared by dilution of the stock standard. The working standards and a blank (water only) were processed with method II as in *Part 2* (starting with deproteination step). Peak areas were plotted *versus* amounts on column.

Six aliquots of the 128 μM standard were processed simultaneously for determination of reproducibility.

HPLC conditions are shown in Table I.

Part 4: Recoveries and reproducibility of spiked serum samples using method II. Working standards (640, 480, 320 and 160 μM) were prepared by dilution of the stock standard with water. Spiked serum samples were prepared by reconstituting serum with the working standards. A blank was prepared by reconstituting with water.

All four spiked samples and blank were diluted 5 times with water. Final concentrations were 128, 96,

TABLE I

HPLC CONDITIONS

Column	MicroPak SP C_8 15 cm × 4 mm
Temperature	Ambient
Mobile phase	0.02 *M* phosphate buffer pH 6.5–acetonitrile (52:48) (can vary by 2%)
Flow-rate	2 ml/min
Detection	Fluorescence, excitation 340 nm, emission 450 nm.
UV	254 nm

[a] Standards were treated similarly for control.

[b] Following Bäck *et al.*'s finding, recoveries of derivatized serum samples improve with dilution [8].

TABLE II

DERIVATIZATION AND SOLID-PHASE EXTRACTION PROCEDURE FOR TOBRAMYCIN STANDARD/SAMPLES

Step	Method I (SPE–Derivatization)	Method II (Derivatization–SPE)
(1) Derivatization for method II		60 μl supernatant was mixed with 240 μl acetonitrile and derivatized with 300 μl OPA reagent[b]
(2) Conditioning	1 ml acetonitrile 1 ml acetonitrile–buffer[a]	1 ml acetonitrile 1 ml acetonitrile–buffer[a]
(3) Load sample	100 μl supernatant mixed with 100 μl acetonitrie/buffer[a] Collect waste	600 μl from step 1 and 300 μl rinse mixed with 100 μl acetonitrile–buffer[a] Collect waste
(4) Wash	200 μl acetonitrile–buffer[a] Collect waste	500 μl acetonitrile–buffer[a] Collect waste
(5) Elute in sample vial Add to sample vial	600 μl acetonitrile 400 μl 0.01 *M* phosphate buffer pH 11	440 μl acetonitrile 40 μl water
(6) Mix	Vortex sample vial	Vortex sample vial
(7)	Dilute waste from steps 3 and 4 with four volumes of water–acetonitrile (20:80) in separate sample vials	
(8) Derivatization for method I	Derivatize 40 μl standard/samples from steps 6 and 7 on AutoSampler with 40 μl OPA reagent	
(9) Inject	25 μl from step 8	25 μl from steps 3, 4 and 6 25 μl from step 1 as calibration standard for both methods

[a] Acetonitrile–0.02 *M* phosphate buffer pH 8 (10:90).
[b] Derivatization is performed manually due to the SPE steps required before injection.

64, 32 and 0 μ*M*. The samples and blank were processed with method II as in *Part 2* (starting with the deproteination step). Relative recoveries were determined based on the 640 μ*M* standard.

Six aliquots of the 128 μ*M* spiked sample were processed simultaneously for determination of reproducibility.

HPLC conditions are shown in Table I.

RESULTS AND DISCUSSION

Part 1

It was found that the derivatization of tobramycin yields two peaks, as shown in Fig. 2. The first peak elutes at 4 min and the second at 10 min. It was noticed that the distribution of the two peaks varies with reaction time as well as the the amount of organic in the reaction medium. The chromatogram in Fig. 2 represents 1 min reaction time and a

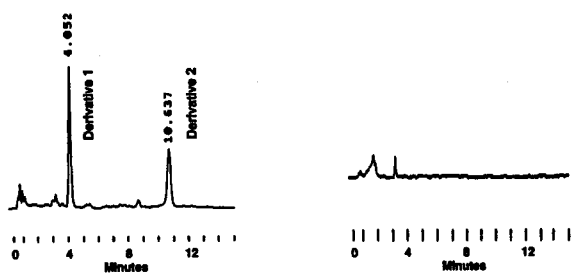

Fig. 2. OPA derivatization of tobramycin. Detection, fluorescence (excitation 340 nm; emission 450 nm).

TABLE III

FACTORS AFFECTING FINAL PRODUCT

Sample solvent	Reaction time (min)	Peak 2/Peak 1[a]
Water	0	0.16
	1	0.17
	10	1.04
	20	2.90
	30	7.17
Acetonitrile–water	0	
0:100		0.16
60:40		0.68
80:20		0.68

[a] Peak 1 for derivative 1, peak 2 for derivative 2.

reaction medium containing 80% acetonitrile. There is no interference from the blank.

The effect of reaction time and reaction medium on the ratio of the area of peak 2/peak 1 is displayed in Table III. The ratio increases with increase in acetonitrile. Precipitation was observed after about 30 min in the low acetonitrile samples, but not at 60 or 80% acetonitrile. To ensure adequate acetonitrile in the sample solvent, 80% acetonitrile was used for the rest of the study. To allow adequate reaction time without making the analysis time too long, a reaction time of 30 min was used.

The difference between detection by UV (254 nm) and detection by fluorescence (excitation 340 nm, emission 450 nm) for OPA-derivatized tobramycin

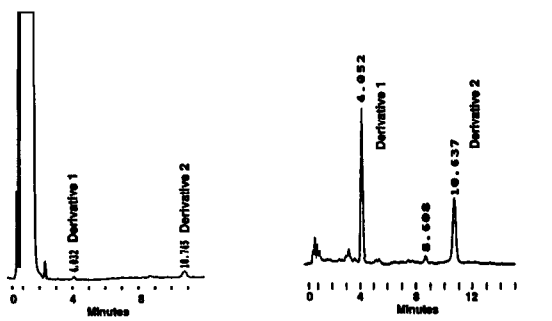

Fig. 3. Detection enhancement using fluorescence *vs.* UV.

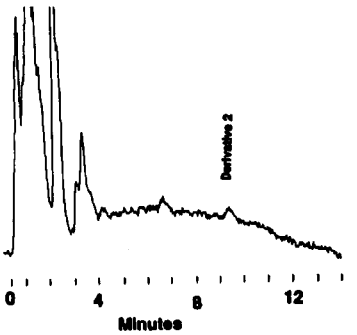

Fig. 4. Detection of OPA-derivatized tobramycin at 10 pmol on column using fluorescence (excitation 340 nm; emission 450 nm).

at 1.6 nmol (on column) is shown in Fig. 3. Fluorescence detection of derivatized tobramycin yields two sharp peaks with a sensitivity about 40 times that of UV detection. The detection limit was found to be 400 pmol (on column) for UV and 10 pmol for fluorescence (Fig. 4).

Peak areas and standard concentrations (peak 2) were linearly related from 0.1 to 1.2 nmol on column with a correlation coefficient of 0.998.

Part 2

The recoveries at each SPE step between methods I and II are shown in Table IV. In method I, where SPE was performed before derivatization, the standard lost 42.7 and 8.2% in the loading and washing steps, and the recovery was 33.7%. The spiked serum sample lost 35.4 and 3.9% in the loading and

TABLE IV

EFFECT OF DERIVATIZATION ON SAMPLE CLEANUP

	Method I, post-SPE derivatization		Method II, pre-SPE derivatization	
	Standard	Spiked serum	Standard	Spiked serum
Sample concentration (μM)	480	480	640	640
Amount on column (pmoles)	300	300	200	200
Loss of sample (%) in				
Loading	42.7	35.4	0.0	0.0
Washing	8.2	3.9	0.0	0.0
Absolute recovery (%)	33.7	2.8	100.3	97.5

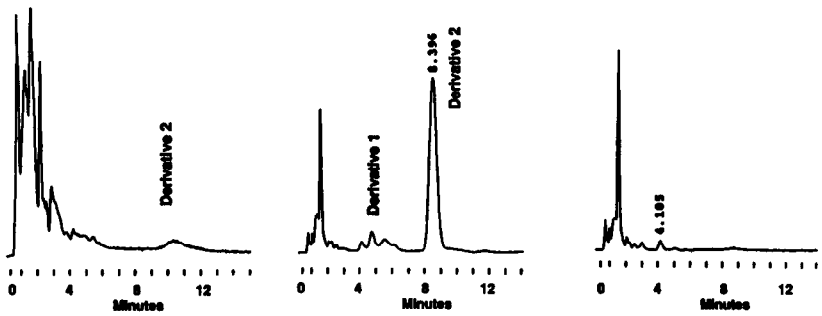

Fig. 5. Chromatograms of tobramycin-spiked serum: method I *vs.* method II. Detection, fluorescence (excitation 340 nm; emission 450 nm).

washing steps and the recovery was 2.8%. The difference between recoveries and loss in SPE is most likely due to protein binding and silanol effects on the SPE columns. On the other hand, using method II, where derivatization was performed before SPE, there was no loss in the loading and washing steps and the recoveries were 100.3% for the standard and 97.5% for the spiked sample.

The chromatograms of the eluates and a blank are shown in Fig. 5[a]. The chromatogram from method II has much less matrix components than that from method I. There is also no interference from the blank.

The derivatization of tobramycin before SPE increased the hydrophobicity of the analyte (relative to the serum matrix) and enhanced its selectivity in the SPE, yielding cleaner chromatograms and higher recoveries than if the derivatization were performed after the SPE.

In method II, SPE was performed immediately after the derivatization. The time lapse between the derivatization and injection is about 15 min for the first sample to be injected. The following samples have a longer time lapse while sitting in the autosampler (15 min of run time for each preceding vial). The reproducibility data show that the difference in time lapse among the samples did not affect the area count. This is possibly due to a stabilization effect when the reaction medium is removed by the SPE (leaving only the pure derivatives).

Part 3

Peak areas and serum concentrations were linearly related, using method II (12.8 to 128 μM corresponding to 20 to 200 pmol on column) with a correlation coefficient of 0.998.

The reproducibility of six standard runs (96 μM) has a relative standard deviation of 1.1%.

Part 4

The recoveries of spiked samples in the concentration range of 160 to 640 μM (32 to 128 μM after dilution) are 92 to 106% with a relative standard deviation of 6.6%.

The reproducibility of 6 spiked samples 640 μM (128 μM after dilution) has an average recovery of 97% and a relative standard deviation of 3.6%.

An improvement that can be visualized for the application of method II is to have an on-line SPE processor to automate the SPE process in addition to the derivatization process and inject the SPE eluate onto the HPLC system.

CONCLUSIONS

The use of fluorescence detection of pre-column

[a] Retention time of derivative 2 is shorter than in *Part I* due to an adjustment in mobile phase to 50% acetonitrile.

derivatized tobramycin has tremendously enhanced detection sensitivity compared to detection by UV. The derivatization is simple, occurs at room temperature, involves no highly toxic reagents and can be fully automated.

Derivatization preceding SPE decreases the polarity of the analyte (tobramycin), thus increasing the selectivity in reversed phase SPE. The results show very good recoveries, linearity, reproducibility and separation of the analyte from the matrix components.

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REFERENCES

1 J. P. Anhalt and S. D. Brown, *Clin. Chem.*, 24 (1978) 1940–1947.
2 H. Fabre, M. Sekkat, M. D. Blanchin and B. Mandrou, *J. Pharm. Biomed. Anal.*, 7 (1989) 1711–1718.
3 D. M. Barends, J. S. Blauw, C. W. Mijnsbergen, C. J. L. R. Grovers and A. Hulshoff, *J. Chromatogr.*, 322 (1985) 321–331.
4 D. M. Barends, J. C. A. M. Brouwers and A. Hulshoff, *J. Pharm Biomed. Anal.*, 5 (1987) 613–617.
5 S. Udenfried, S. Stein, P. Boehler, W. Dairman, W. Leingruber and M. Weigele, *Science (Washington, D.C.)*, 178 (1972) 871–872.
6 M. Roth, *Anal. Chem.*, 43 (1971) 880–882.
7 M. W. Dong, *LC · GC*, 5 (1987) 255–260.
8 S. E. Bäck, I. Nilsson-Ehle and P. Nilsson-Ehle, *Clin Chem.*, 25 (1979) 1222–1225.
9 J. D'Souza and R. I. Ogilvie, *J. Chromatogr.*, 232 (1982) 212–218.
10 L. Essers, *J. Chromatogr.*, 305 (1984) 345–352.
11 J. Bell, R. Simpson and A. Mayer, *Am. Lab.*, May (1987) 106–110.